

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 15/51, 15/19, A61K 69/29</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 93/15207</b> <b>(43) International Publication Date:</b> <b>5 August 1993 (05.08.93)</b>
<b>(21) International Application Number:</b> <b>PCT/US93/01009</b> <b>(22) International Filing Date:</b> <b>4 February 1993 (04.02.93)</b>  <b>(30) Priority data:</b> <b>07/830,417 4 February 1992 (04.02.92) US</b>  <b>(71) Applicant: VIAGENE, INC. [US/US]; 11075 Roselle Street, San Diego, CA 92121 (US).</b>  <b>(72) Inventors: JOLLY, Douglas, J. ; 3050H Via Alicante Drive, La Jolla, CA 92037 (US). CHANG, Stephen, M., W. ; 9838 Via Caecras, San Diego, CA 92129 (US). LEE, William, Tsung-Liang ; 7961 Calle Posada, Carlsbad, CA 92009 (US). TOWNSEND, Kay ; 926 Birchview Drive, Encinitas, CA 92024 (US). O'DEA, Joann ; 8842 Cliff-ridge Avenue, LaJolla, CA 92037 (US).</b>		<b>(74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).</b>  <b>(81) Designated States: AU, CA, CZ, JP, KP, KR, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b>  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title: HEPATITIS THERAPEUTICS</b>  <b>(57) Abstract</b>  The present invention provides a method of treating hepatitis B infections comprising the step of administering a vector construct which directs the expression of at least one immunogenic portion of a hepatitis B antigen, such that an immune response is generated. Also provided are methods for treating hepatitis C infections, as well as hepatocellular carcinomas.		

**BEST AVAILABLE COPY**

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Uzbekistan	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

Description

## HEPATITIS THERAPEUTICS

5

Technical Field

The present invention relates generally to methods for treating hepatitis, as well as hepatitis-associated carcinomas.

10 Background of the Invention

Hepatitis is a systemic disease which predominantly affects the liver. The disease is typified by the initial onset of symptoms such as anorexia, nausea, vomiting, fatigue, malaise, arthralgias, myalgias, and headaches, followed by the onset of jaundice. The disease may also be characterized by increased serum  
15 levels of the aminotransferases AST and ALT. Quantification of these enzymes in serum indicates the extent of liver damage.

There are five general categories of viral agents which have been associated with hepatitis: the hepatitis A virus (HAV); the hepatitis B virus (HBV); two types of non-A, non-B (NANB) agents, one blood-borne (hepatitis C)  
20 and the other enterically transmitted (hepatitis E); and the HBV-associated delta agent (hepatitis D).

There are two general clinical categories of hepatitis, acute hepatitis and chronic hepatitis. Symptoms for acute hepatitis range from asymptomatic and non-apparent to fatal infections. The disease may be subclinical and persistent, or  
25 rapidly progress to chronic liver disease with cirrhosis, and in some cases, to hepatocellular carcinoma. Acute hepatitis B infection in adult Caucasians in the United States progresses to chronic hepatitis B in about 5% to 10% of the cases. In the remainder of the cases, approximately 65% are asymptomatic. In the Far East, infection is usually perinatal, and 50% to 90% progress to the chronic state.  
30 It is likely that the different rates of progression are linked to the age at infection rather than genetic differences in the hosts. In the United States, about 0.2% of the population is chronically infected, with higher percentages in high-risk groups such as physicians, drug addicts and renal dialysis patients. In countries such as Taiwan, Hong Kong and Singapore, the level in the population with hepatitis  
35 infection may be as high as 10%.

In the United States, about 20% of patients with chronic hepatitis die of liver failure, and a further 5% develop hepatitis B-associated carcinoma. In

the Far East, a large percentage of the population is infected with HBV, and after a long chronic infection (20 to 40 years), approximately 25% of these will develop hepatocellular carcinoma.

- After the development of serologic tests for both hepatitis A and B, investigators identified other patients with hepatitis-like symptoms, and with incubation periods and modes of transmission consistent with an infectious disease, but without serologic evidence of hepatitis A or B infection. After almost 15 years, the causative agent was identified as an RNA virus. This virus (designated "hepatitis C") has no homology with HBV, retroviruses, or other hepatitis viruses.

- Hepatitis C (HCV) appears to be the major cause of post-transfusion and sporadic non-A, non-B (NANB) hepatitis worldwide, and plays a major role in the development of chronic liver disease, including hepatocellular carcinoma (Kuo et al., *Science* 244:362-364, 1989; Choo et al., *British Medical Bulletin* 46(2):423-441, 1990). Of the approximately 3 million persons who receive transfusions each year, approximately 150,000 will develop acute hepatitis C (Davis et al., *New Eng. J. Med.* 321(22):1501-1506, 1989). In addition, of those that develop acute hepatitis C, at least one-half will develop chronic hepatitis C.

- Until recently, no therapy has proven effective for treatment of acute or chronic hepatitis B or C infections, and patients infected with hepatitis must generally allow the disease run its course. Most anti-viral drugs, such as acyclovir, as well as attempts to bolster the immune system through the use of corticosteroids have proven ineffective (Alter, "Viral hepatitis and liver disease," Zuckerman (ed.), New York: Alan R. Liss, pp. 537-42, 1988). Some anti-viral activity has been observed with adenosine arabinoside (Jacyna et al., *British Med. Bull.* 46:368-382, 1990), although toxic side effects which are associated with this drug render such treatment unacceptable.

- One treatment that has provided some benefit for chronic hepatitis B and C infections is the use of recombinant alpha interferon (Davis et al., *New Eng. J. Med.* 321(22):1501-1506, 1989; Perrillo et al., *New Eng. J. Med.* 323:295-301, 1990). However, for patients with hepatitis B infections only about 35% of infectees responded to such treatment, and in perinatal infectees only about 10% responded to treatment. For hepatitis C infections, despite apparent short-term success utilizing such therapy, six months after termination of treatment half of the patients who responded to therapy had relapsed. In addition, a further difficulty with alpha interferon therapy is that the composition frequently has toxic side effects which require reduced dosages for sensitive patients.

Therefore, therapeutics that could serve to augment natural host defenses against hepatitis, or against tumor induction and progression, with reduced cytotoxicity, or that allows treatment of interferon non-responsive individuals would be very beneficial. The present invention provides such  
5 therapeutic agents, and further provides other related advantages.

#### Summary of the Invention

Briefly stated, the present invention is directed toward methods of treating hepatitis B and hepatitis C infections, as well as hepatocellular  
10 carcinomas (HCC). Within one aspect of the present invention, a method is provided for treating hepatitis B infections in warm-blooded animals comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis B antigen, such that an immune response is generated. Within other aspects of the  
15 invention, an immunomodulatory cofactor may also be administered, or expressed along with an immunogenic portion of a hepatitis B antigen. Within various embodiments, the vector construct directs the expression of HBeAg, HBcAg, HBsAg, ORF 5, ORF 6, the HBV pol antigen, or any combination of these antigens. Within one embodiment the HBsAg is selected from the group  
20 consisting of S, pre-S1, and pre-S2.

Within a related aspect of the present invention, a vector construct is provided which directs the co-expression of at least one immunogenic portion of a hepatitis B antigen and an immunomodulatory cofactor. Also provided are pharmaceutical compositions comprising the recombinant viruses in combination  
25 with a pharmaceutically acceptable carrier or diluent.

Within another aspect of the present invention, a method is provided for destroying hepatitis B carcinoma cells in warm-blooded animals, comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of antigen X such that an  
30 immune response is generated. Within other aspects of the invention, an immunomodulatory cofactor may also be administered, or expressed along with, the immunogenic portion of antigen X.

Within yet another aspect of the invention, a vector construct is provided which directs the expression of an immunogenic portion of antigen X, or  
35 co-expresses this antigen with an immunomodulatory cofactor. Also provided are pharmaceutical compositions comprising these recombinant viruses in combination with a pharmaceutically acceptable carrier or diluent.

Within a further aspect of the present invention, a method of treating hepatitis C infections in warm-blooded animals is provided, comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis C antigen such that an immune response is generated. Within other aspects, an immunomodulatory cofactor may also be administered or co-expressed with the immunogenic portion of a hepatitis C antigen. Within various embodiments, the vector construct may express the core antigen C, antigen E1, antigen E2/NS1, antigen NS2, antigen NS3, antigen NS4, antigen NS5, an S antigen or combinations thereof.

Within a related aspect of the invention, a vector construct is provided which directs the expression of at least one immunogenic portion of a hepatitis C antigen, or co-expresses this antigen in combination with an immunomodulatory cofactor. Within another embodiment, a vector construct is provided which directs the co-expression of at least one immunogenic portion of a hepatitis B antigen and at least one immunogenic portion of a hepatitis C antigen. Also provided are pharmaceutical compositions comprising the recombinant viruses in combination with a pharmaceutically acceptable carrier or diluent.

Within another aspect of the present invention, a method is provided for destroying hepatitis C carcinoma cells in warm-blooded animals, comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of the polyprotein antigen, such that an immune response is generated. Within other aspects of the invention, an immunomodulatory cofactor may also be administered, or expressed along with an immunogenic portion of a hepatitis C antigen.

Within a related aspect of the invention, a vector construct is provided which directs the expression of an immunogenic portion of the polyprotein antigen, or co-expresses this antigen with an immunomodulatory cofactor. Also provided are pharmaceutical compositions comprising these recombinant viruses in combination with a pharmaceutically acceptable carrier or diluent.

Within a further aspect of the present invention, a method is provided for treating chronic hepatitis infections in warm-blooded animals, comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis B antigen, and at least one immunogenic portion of a hepatitis C antigen, such that an immune response is generated.

Vector constructs of the present invention may be delivered in a variety of ways, including for example by a recombinant retrovirus, or a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus (e.g., the canary pox virus or the vaccinia virus), influenza virus, adenovirus, parvovirus (e.g., the adeno-associated virus, B19 or MVN), herpes virus, SV40, HIV, measles and alpha viruses such as the Sindbis virus and corona virus. In addition, the vector construct, or nucleic acids which encode the relevant immunogenic portion, may be administered to a patient directly, for example by transfection methods such as lipofection, direct DNA injection, microprojectile bombardment, liposomes, CaPO<sub>4</sub>, or DNA ligand. The present invention also provides compositions (including, for example, various adjuvants) and methods suitable for administering the immunogenic proteins themselves, vector constructs, retroviral vectors, or retroviral vectors along with immunomodulatory cofactors.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

#### Brief Description of the Drawings

Figure 1 is a schematic illustration which outlines the recovery of Hepatitis B e sequence from ATCC 45020.

Figure 2 is a diagrammatic representation of the nucleotide sequence of HBV (*adw*) precore/core (SEQ ID. NO. 56) and the corrected sequences.

Figure 3 is a schematic representation of the correction of the deletion in HBe-c sequence from pAM6 (ATCC 45020).

Figure 4 is a DNA sequencing gel showing the corrected nucleotide sequences from SK<sup>+</sup>HBe-c.

Figure 5 is a chart showing expression of HBVe protein from the following retrovirally transduced murine cell lines BC10ME, Bl/6, L-M(TK<sup>-</sup>), EL4, and retrovirally transduced EBV-transformed human B-cell line, JY-LCL, as determined by ELISA.

Figure 6 is a Western blot showing expression of p17 kD HBV e protein secreted by retrovirally transduced BC10ME and Bl/6 cells and p22 and p23 kD precore intermediate proteins in cell lysates from retrovirally transduced BC10ME and Bl/6 cells.

Figure 7 is a graph showing induction of antibody responses against HBVe antigen in Balb/C and C57Bl/6 mice injected with syngeneic cells

expressing the antigen or by direct injection with the retroviral vector encoding HBVe antigen.

Figure 8 is a diagrammatic representation of vector construct KT-HBV core/B7/BB1 which expresses both HBV core and B7/BB1 proteins.

#### Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first define certain terms that will be used hereinafter. All references which have been cited below are hereby incorporated by reference in their entirety.

"Immunogenic portion" as utilized within the present invention, refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (*i.e.*, cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Representative assays which may be utilized to determine immunogenicity (*e.g.*, cell-mediated immune response), are described in more detail below, as well as in Example 12. Cell mediated immune responses may be mediated through Major Histocompatibility Complex ("MHC") class I presentation, MHC Class II presentation, or both.

"Vector construct" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The vector construct must include promoter elements and preferably includes a signal that directs poly-adenylation. In addition, the vector construct must include a sequence which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. Preferably, the vector construct also includes a selectable marker such as Neo, SV<sub>2</sub> Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the vector construct is placed into a retrovirus, the vector construct must include a packaging signal and long terminal repeats (LTRs) appropriate to the retrovirus used (if these are not already present).

"Immunomodulatory cofactor" refers to factors which, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the cofactor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro*



assays which measure cellular proliferation (e.g.,  $^3\text{H}$  thymidine uptake), and *in vitro* cytotoxic assays (e.g., which measure  $^{51}\text{Cr}$  release) (see, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). Immunomodulatory cofactors may be active both *in vivo* and *ex vivo*. Representative examples of such cofactors include cytokines, such as interleukins 2, 4, and 6 (among others), alpha interferons, beta interferons, gamma interferons, GM-CSF, G-CSF, and tumor necrosis factors (TNFs). Other immunomodulatory cofactors include, for example, CD3, ICAM-1, ICAM-2, LFA-1, LFA-3, MHC class I molecules, MHC class II molecules, B7/BB1,  $\beta_2$ -microglobulin, chaperones, or analogs thereof.

As noted above, the present invention is directed towards methods and compositions for treating hepatitis B and C infections, as well as hepatocellular carcinomas. Briefly, the ability to recognize and defend against foreign pathogens is central to the function of the immune system. This system, through immune recognition, is capable of distinguishing "self" from "nonself" (foreign), which is essential to ensure that defensive mechanisms are directed towards invading entities rather than against host tissues. The methods which are described in greater detail below provide an effective means of inducing potent class I-restricted protective and therapeutic CTL responses, as well as humoral responses.

As noted above, within one aspect of the present invention, a method for treating hepatitis B infections in warm-blooded animals is provided, comprising the step of administering a vector construct to a warm-blooded animal which directs the expression of at least one immunogenic portion of a hepatitis B antigen, such that an immune response is generated.

Briefly, the hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length, and has been well characterized (Tiollais et al., *Science* 213:406-411, 1981; Tiollais et al., *Nature* 317:489-495, 1985; and Ganem and Varmus, *Ann. Rev. Biochem.* 56:651-693, 1987). The hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (see Blum et al., "The Molecular Biology of Hepatitis B Virus," *TIG* 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of P22 precore intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

The HBsAgs (designated "large," "middle," and "small") are encoded by three regions of the hepatitis B genome: S, pre-S2 and pre-S1. The large

protein, which has a length varying from 389 to 400 amino acids, is encoded by pre-S1, pre-S2, and S regions, and is found in glycosylated and non-glycosylated forms. The middle protein is 281 amino acids long and is encoded by the pre-S2 and S regions. The small protein is 226 amino acids long and is encoded by the S region. It exists in two forms, glycosylated (GP 27<sup>s</sup>) and non-glycosylated (P24<sup>s</sup>). If each of these regions are expressed separately, the pre-S1 region will code for a protein of approximately 119 amino acids, the pre-S2 region will code for a protein of approximately 55 amino acids, and the S region will code for a protein of approximately 226 amino acids.

As will be evident to one of ordinary skill in the art, various immunogenic portions of the above described S antigens may be combined in order to present an immune response when administered by one of the vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants however have also been identified by two-dimensional double immunodiffusion (Ouchterlony, *Progr. Allergy* 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, *J. Infect.* 123:671, 1971; Bancroft et al., *J. Immunol.* 109:842, 1972; Courouge et al., *Bibl. Haematol.* 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S open reading frame: (1) exchange of lysine-122 to arginine in the hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In black Africa, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw<sub>2</sub> is more abundant (*Molecular Biology of the Hepatitis B Virus*, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected liver. The

polymerase protein consists of at least two domains: the amino terminal domain encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g.,  
5 below and in Examples 12Aii and 13), expressed utilizing vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens such as ORF 5 and ORF 6, (Miller et al., *Hepatology* 9:322-327, 1989), may be expressed utilizing vector constructs as described herein. Representative examples of vector  
10 constructs utilizing ORF 5 and ORF 6 are set forth below in Examples 2J, 2K and 5H, 5I.

Molecularly cloned genomes which encode the hepatitis B virus may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020  
15 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (see Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981). (Note that, as described in Example 2A and as shown in Figure 2, correctable errors occur in the sequence of ATCC No. 45020.)

As noted above, at least one immunogenic portion of a hepatitis B  
20 antigen is incorporated into a vector construct. The immunogenic portion(s) which are incorporated into the vector construct may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long, and may include the entire antigen. Immunogenicity of a particular sequence is  
25 often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITIS (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay, such as that described in Example 13. Other assays, however, may also be utilized,  
30 including, for example, ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays. A particularly preferred assay is described in more detail below in Example 12B.

Immunogenic portions may also be selected by other methods. For  
35 example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the

same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med.* 173:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Particularly preferred immunogenic portions for incorporation into vector constructs include HBcAg, HBsAg, and HBsAgs as described in greater detail below in the Examples.

Additional immunogenic portions of the hepatitis B virus may be obtained by truncating the coding sequence at various locations including, for example, the following sites: Bst UI, Ssp I, Ppu M1, and Msp I (Valenzuela et al., *Nature* 280:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B. N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic). Further methods for determining suitable immunogenic portions as well as methods are also described below in the context of hepatitis C.

As noted above, more than one immunogenic portion may be incorporated into the vector construct. For example, a vector construct may express (either separately or as one construct) all or portions of HBcAg, HBsAg, HBsAgs, HBxAg as well as immunogenic portions of HCV antigens as discussed below. In addition, the vector construct may also co-express an immunomodulatory cofactor, such as alpha interferon (Finter et al., *Drugs* 42(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz.* 78:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA* 86:2046-2050, 1989; Faktor et al., *Oncogene* 5:867-872, 1990), beta interferon (Seif et al., *J. Virol.* 65:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 20082015, 1991; Watanabe et al., *PNAS* 86:9456-9460, 1989; Gansbacher et al., *Cancer Research* 50:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother.* 30:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), TNFs (Jayaraman et al., *J. Immunology* 144:942-951, 1990), Interleukin-2 (IL-2) (Karupiah et al., *J. Immunology* 144:290-298, 1990; Weber et al., *J. Exp. Med.* 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med.* 172:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-4 (Tepper et al., *Cell* 57:503-512, 1989; Gohumbek et al., *Science* 254:713-716, 1991; U.S. Patent No. 5,017,691), IL-6 (Brakenhof et al., *J. Immunol.* 139:4116-4121, 1987; WO 90/06370), ICAM-1 (Altman et al., *Nature* 338:512-514, 1989), ICAM-2, LFA-1, LFA-3, MHC class I

molecules, MHC class II molecules,  $\beta_2$ -microglobulin, chaperones, CD3, B7/BB1, MHC linked transporter proteins or analogs thereof.

The choice of which immunomodulatory cofactor to include within a vector construct may be based upon known therapeutic effects of the cofactor, or, experimentally determined. For example, in chronic hepatitis B infections alpha interferon has been found to be efficacious in compensating a patient's immunological deficit, and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory cofactor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA matched cells (e.g., EBV transformed cells), and transduced with a vector construct which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory cofactor. Stimulated PBLs are used as effectors in a CTL assay with the HLA matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory cofactor.

Molecules which encode the above-described immunomodulatory cofactors may be obtained from a variety of sources. For example, plasmids which contain these sequences may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC No. 57592 (which contains sequences encoding Interleukin-4), and ATCC 67153 (which contains sequences encoding Interleukin-6).

In a similar manner, sequences which encode immunomodulatory cofactors may be readily obtained from cells which express or contain sequences which encode these cofactors. Briefly, within one embodiment, primers are prepared on either side of the desired sequence, which is subsequently amplified by PCR (see U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159) (see also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.),

Stockton Press, 1989). In particular, a double-stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode immunomodulatory cofactors may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (e.g., ABI DNA synthesizer model 392 (Foster City, California)). Such sequences may also be linked together through complementary ends, followed by PCR amplification (Vent polymerase, New England Biomedical, Beverly, Massachusetts) to form long double-stranded DNA molecules (Foguet et al., *Biotechniques* 13:674-675, 1992).

Once an immunogenic portion(s) (and, if desired, an immunomodulatory cofactor) have been selected, genes which encode these proteins are placed into a vector construct which directs their expression. In general, such vectors encode only these genes, and no selectable marker. Vectors encoding and leading to expression of a specific antigen and immunomodulatory cofactor may be readily constructed by those skilled in the art. In particular, construction of vector constructs as well as administration of retroviral constructs by direct injection is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603, filed September 21, 1990). These vector constructs may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see U.S.S.N. 07/800,921) in order to generate producer cell lines for the production of retroviral vector particle which are replication incompetent.

Various assays may be utilized in order to detect the presence of any replication competent infectious retroviruses. One preferred assay is the extended S<sup>+</sup>L<sup>-</sup> assay described in Example 8.

Within a particularly preferred embodiment, vector constructs may be constructed to include a promoter such as SV40 (see Kriegler et al., *Cell* 38:483, 1984), cytomegalovirus ("CMV") (see Boshart et al., *Cell* 41:521-530, 1991), or an internal ribosomal binding site ("IRBS"). Briefly, with respect to IRBS, the five prime untranslated region of the immunoglobulin heavy chain binding protein has been shown to support the internal engagement of a bicistronic message (see Jacejak and Sarnow, *Nature* 353:90-94, 1991). This sequence is small (300 bp), and may readily be incorporated into a retroviral vector in order to express multiple genes from a multi-cistronic message whose cistrons begin with this

sequence. A representative vector construct utilizing IRBS is set forth in more detail below in Examples 6C and 6D.

In addition, vector constructs may also be developed and utilized with other viral carriers including, for example, poliovirus (Evans et al., *Nature* 339:385-388, 1989; and Sabin, *J. Biol. Standardization* 1:115-118, 1973); rhinovirus (Arnold, *J. Cell. Biochem.* 1401-405, 1990); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112 and 4,769,330; WO 89/01973); SV40 (Mulligan et al., *Nature* 277:108-114, 1979); influenza virus (Luytjes et al., *Cell* 59:1107-1113, 1989; McMichael et al., *N. Eng. J. Med.* 309:13-17, 1983; and Yap et al., *Nature* 273:238-239, 1978); adenovirus (Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991); parvovirus such as adeno-associated virus (Samulski et al., *J. Vir.* 63:3822-3828, 1989; Mendelson et al., *Viol.* 166:154-165, 1988); herpes (Kit, *Adv. Exp. Med. Biol.* 215:219-236, 1989); SV40; HIV (Poznansky, *J. Virol.* 65:532-536, 1991); measles (EP 0 440,219); Sindbis virus (Xiong et al., *Science* 234:1188-1191, 1989); and corona virus.

Once a vector construct has been prepared, it may be administered to a warm-blooded animal in order to treat a hepatitis B infection. Methods for administering a vector construct via a retroviral vector (such as by direct injection of the retroviral construct) are described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603). Such methods include, for example, transfection by methods utilizing various physical methods, such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); liposomes (Wang et al., *PNAS* 84:7851-7855, 1987);  $\text{CaPO}_4$  (Dubensky et al., *PNAS* 81:7529-7533, 1984); or DNA ligand (Wu et al., *J. Biol. Chem.* 264:16985-16987, 1989). In addition, the vector construct, or nucleic acids which encode the relevant immunogenic portion, may be administered to a patient directly, for example by transfection methods such as lipofection, direct DNA injection, microprojectile bombardment, liposomes,  $\text{CaPO}_4$ , or DNA ligand. Compositions and methods suitable for administering immunogenic proteins themselves, vector constructs, viral vectors, or viral vectors along with immunomodulatory cofactors, are discussed in more detail below.

Within another aspect of the present invention, a method is provided for treating hepatitis C infections, comprising the step of administering

to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis C antigen, such that an immune response is generated. Briefly, as noted above, hepatitis C (non-A, non-B (NANB) hepatitis) is a common disease that accounts for more than 90% of the cases of hepatitis that develop after transfusion (Choo et al., *Science* 244:359-362, 1989). Most information on NANB hepatitis was derived from chimpanzee transmission studies which showed that NANB hepatitis was present in most human infections at titers of only  $10^2$  -  $10^3$  CID/ml (chimp infectious doses per ml). In addition, the disease was found to cause the appearance of distinctive, membranous tubules within the hepatocytes of experimentally infected chimpanzees. This "tubule-forming" agent was subsequently termed hepatitis C virus (HCV).

The genomic RNA of HCV has recently been determined to have a sequence of 9379 nucleotides (Choo et al., *Proc. Natl. Acad. Sci. USA* 88:2451-2455, 1991; Choo et al., *Brit. Med. Bull.* 46(2):423-441, 1990; Okamoto et al., *J. Gen. Vir.* 72:2697-2704, 1991; see also Genbank Accession No. M67463, Intelligenetics (Mountain View, California). The sequence encodes a polyprotein precursor of 3011 amino acids, which has significant homology to proteins of the flavivirus family. The polyprotein is believed to contain several different viral proteins, including C (nucleocapsid protein) E1, E2/NS1, and non-structural proteins NS2, NS3, NS4, and NS5 (Houghton et al., *Hepatology* 14:381-388, 1991).

As noted above, within one embodiment of the present invention, at least one immunogenic portion of a hepatitis C antigen is incorporated into a vector construct. Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology* 14:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polyprotein may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict T cell epitopes, which frequently possess an immunogenic amphipathic alpha-helix. T cell epitopes may be predicted utilizing computer algorithms such as TSites (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. This analysis is primarily based upon (1) structural properties of the proteins (principally alpha-helical periodicity and amphipathicity), and (2) motifs found in sequences recognized by MHC Class I and Class II molecules. In general



however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA which detects the presence of antibodies against a newly introduced vector, Example 12B, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays as described in Example 12C. A particularly preferred assay is described in more detail below in Example 12Ai.

Immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, *op. cit.*, Millich et al., *Proc. Natl. Acad. Sci. USA* 85:1610-1614, 1988; Willis, *Nature* 340:323-324, 1989; Griffiths et al., *J. Virol.* 65:450-456, 1991).

Preferred immunogenic portions may also be selected in the following manner. Briefly, blood samples from a patient with HCV are analyzed with antibodies to individual HCV polyprotein regions (e.g., HCV core, E1, E2/SNI and NS2-NS5 regions), in order to determine which antigenic fragments are present in the patient's serum. In patients treated with alpha interferon to give temporary remission, some antigenic determinants will disappear and be supplanted by endogenous antibodies to the antigen. Such antigens are useful as immunogenic portions within the context of the present invention (Hayata et al., *Hepatology* 13:1022-1028, 1991; Davis et al., *N. Eng. J. Med.* 321:1501-1506, 1989).

Once at least one immunogenic portion of hepatitis C (and, if desired, immunomodulatory cofactors and/or immunogenic portions of HBV as discussed above) has been selected, it may be placed into a vector construct which directs its expression. As described above for hepatitis B therapeutics, various recombinant viral vectors may be utilized to carry the vector construct including, for example, recombinant retroviruses (*see*, U.S.S.N. 07/586,603). In addition, as noted above, vector constructs may be developed and utilized with other viral carriers including, for example, poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles, Sindbis virus and corona virus. In addition, the vector construct, or nucleic acids which encode the relevant immunogenic portion, may be administered to a patient directly, for example by transfection methods such as lipofection, direct DNA injection, microprojectile bombardment, liposomes, CaPO<sub>4</sub>, or DNA ligand. Compositions and methods suitable for

administering immunogenic proteins themselves, vector constructs, viral vectors, or viral vectors along with immunomodulatory cofactors, are discussed in more detail below.

Within other aspects of the present invention, methods are provided  
5 for destroying hepatoma cells. Briefly, hepatocellular carcinoma is the most common cancer worldwide. It is responsible for approximately 1,000,000 deaths annually, most of them in China and in sub-Saharan Africa. There is strong evidence of an etiologic role for hepatitis B infection in hepatocellular carcinoma. Carriers of the HBV are at greater than 90 times higher risk for hepatocellular  
10 carcinoma than noncarriers. In many cases, hepatitis B virus DNA is integrated within the cellular genome of the tumor. Similarly, hepatitis C virus has also recently been determined to be associated with hepatocellular carcinoma, based upon the observation that circulating HCV antibodies can be found in some patients with hepatocellular carcinoma. At present, surgical resection offers the  
15 only treatment for hepatocellular carcinoma, as chemotherapy, radiotherapy, and immunotherapy have not shown much promise (Colombo et al., *Lancet* 1006-1008, October 28, 1989; Bisceglie et al., *Ann. of Internal Med.* 108:390-401, 1988; Watanabe et al., *Int. J. Cancer* 48:340-343, 1991; Bisceglie et al., *Amer. J. Gastro.* 86:335-338, 1991).

Within another aspect of the present invention, a method is  
20 provided for destroying hepatitis B carcinoma cells comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated. Sequences which encode the HBxAg may readily be  
25 obtained by one of skill in the art given the disclosure provided herein. Briefly, within one embodiment of the present invention, a 612 bp Nco I-Sal I is recovered from ATCC 45020, and inserted into vector constructs as described above for other hepatitis B antigens.

The X antigen, however, is a known transactivator which may  
30 function in a manner similar to other potential oncogenes (e.g., E1A). Thus, it is generally preferable to first alter the X antigen such that the gene product is non-tumorigenic before inserting it into a vector construct. Various methods may be utilized to render the X antigen non-tumorigenic including, for example, by  
35 truncation, point mutation, addition of premature stop codons, or phosphorylation site alteration. Within one embodiment, the sequence or gene of interest which encodes the x antigen is truncated. Truncation may produce a variety of fragments, although it is generally preferable to retain greater than or equal to

50% of the encoding gene sequence. In addition, it is necessary that any truncation leave intact some of the immunogenic sequences of the gene product. Alternatively, within another embodiment of the invention, multiple translational termination codons may be introduced into the gene which encodes the altered X antigen. Insertion of termination codons prematurely terminates protein expression, thus preventing expression of the transforming portion of the protein.

The X gene or modified versions thereof may be tested for tumorigenicity in a variety of ways. Representative assays include tumor formation in nude mice (see Example 14A), colony formation in soft agar (see Example 14B), and preparation of transgenic animals, such as transgenic mice.

Tumor formation in nude mice or rats is a particularly important and sensitive method for determining tumorigenicity. Nude mice lack a functional cellular immune system (i.e., do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if infected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the vector construct is administered to syngeneic murine cells, and the cells injected into nude mice. The mice are visually examined for a period of 4 to 16 weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovarella et al., *J. Natl. Cancer Inst.* 48:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of biological drugs," *Abnormal Cells, New Products and Risk*, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; Levenbook et al., *J. Biol. Std.* 13:135-141, 1985).

Tumorigenicity may also be assessed by visualizing colony formation in soft agar (MacPherson and Montagnier, *Virology* 23:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is anchorage-dependent growth. More specifically, normal non-tumorigenic cells will stop proliferation when they are plated in a semi-solid agar support medium, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an immunogenic portion of antigen X (Stewart et al., *Cell* 38:627-637, 1984; Quaife et al., *Cell* 48:1023-1034, 1987; Koike et al., *Proc. Natl. Acad. Sci. USA* 86:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all tissues of the animal (see generally, WO

90/08832). This dysregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

As noted above, once an immunogenic portion of antigen X has been selected (which is preferably non-tumorigenic), it may be inserted into a vector construct as described above, and carried by a recombinant virus. As noted above, vector constructs of the present invention may be carried in a variety of ways including, for example, by a recombinant retrovirus, or a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles, corona and Sindbis virus. In addition, the vector construct, or nucleic acids which encode the relevant immunogenic portion, may be administered to a patient directly, for example, by transfection methods such as lipofection, direct DNA injection, microprojectile bombardment, liposomes,  $\text{CaPO}_4$ , or DNA ligand. Compositions and methods suitable for administering immunogenic proteins themselves, vector constructs, viral vectors, or viral vectors along with immunomodulatory cofactors, are discussed in more detail below.

Within another aspect of the present invention, a method is provided for destroying hepatitis C carcinoma cells comprising the step of administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of a hepatitis C antigen. Preferred immunogenic portion(s) of a hepatitis C antigen may be found in the polyprotein which contains the Core antigen and the NS1-NS5 regions (Choo et al., *Proc. Natl. Acad. Sci. USA* 88:2451-2455, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above preferred immunogenic portions may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict T cell epitopes, which frequently possess an immunogenic amphipathic alpha-helix. Another method that may also be utilized to predict immunogenic portions is to determine which portion has the property of CTL induction in mice utilizing retroviral vectors (see, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). As noted within Warner et al., CTL induction in mice may be utilized to predict cellular immunogenicity in humans. Preferred immunogenic portions may also be deduced by determining which fragments of the polyprotein antigen or peptides are capable of causing lysis by autologous patient lymphocytes of target cells (e.g., EBV transduced

lymphocytes) expressing the fragments after vector transduction of the corresponding genes (Example 13).

As noted above, once an immunogenic portion has been selected, it is generally preferable to ensure that it is non-tumorigenic. This may be accomplished by a variety of methods, including for example by truncation, point mutation, addition of premature stop codons, or phosphorylation site alteration. The polypeptide antigen or modified version thereof may also be tested for tumorigenicity utilizing the above-described methods, or by the methods described in Example 14.

Immunogenic portion(s) (as well as immunomodulatory cofactors, if desired) may then be inserted into a vector construct, and carried by a recombinant virus as described above. Additionally, as should be evident to one of ordinary skill in the art, vectors as described above for the treatment of acute and chronic HCV infection may also be utilized to treat hepatocellular carcinoma linked HCV infections. Compositions and methods suitable for administering the immunogenic proteins themselves, vector constructs, viral vectors, or viral vectors along with immunomodulatory cofactors, are discussed in more detail below.

Within another aspect of the present invention, vector constructs may be prepared which direct the co-expression of several of the above described immunogenic portions (as well as immunomodulatory co-factors, if desired). For example, within one embodiment vector constructs may be prepared which direct the co-expression of both an immunogenic portion of the hepatitis B antigen, as well as an immunogenic portion of the hepatitis C polyprotein. Such constructs may be administered as described above and below, in order to prevent or treat acute and chronic hepatitis infections of either type B or C. Similarly, within other embodiments vector constructs may be prepared which direct the co-expression of both an immunogenic portion of the hepatitis B X antigen, as well as an immunogenic portion of the hepatitis C polyprotein. Such constructs may similarly be administered in order to treat hepatocellular carcinoma of which is associated with either hepatitis B or C. In addition, because those individuals chronically infected with hepatitis B and C are at higher risk for developing hepatocellular carcinoma, such a vector may also be utilized as a prophylactic treatment for the disease.

As noted above, various methods may be utilized to administer vector constructs of the present invention, or nucleic acids which encode the immunogenic portion(s) discussed above, to warm-blooded animals such as humans, directly (Curiel et al., *Human Gene and Therapy* 3:147-154, 1992).

In addition, an immune response (including CTL) may also be generated by administration of a bacteria which expresses the immunogenic portion(s) discussed above on its cell surface. Representative examples include BCG (Stover, *Nature* 351:456-458, 1991) and salmonella (Newton et al., *Science* 244:70-72, 1989).

Cell mediated and humoral responses may also be induced against hepatitis by parenteral administration of the immunogenic portion(s) discussed above. Briefly, immunogenic portions carrying relevant epitopes can be produced in a number of known ways (Ellis and Gerety, *J. Med. Virol.* 31:54-58, 1990), including chemical synthesis (Bergot et al., *Applied Biosystems Peptide Synthesizer User Bulletin No. 16*, 1986, Applied Biosystems, Foster City California) and DNA expression in recombinant systems, such as the insect-derived baculovirus system (Doerfler, *Current Topics in Immunology* 131:51-68, 1986), mammalian-derived systems (such as CHO cells) (Berman et al., *J. Virol.* 63:3489-3498, 1989), yeast-derived systems (McAleer et al., *Nature* 307:178-180), and prokaryotic systems (Burrell et al., *Nature* 279:43-47, 1979).

The proteins or peptides may then be purified by conventional means and delivered by a number of methods to induce cell-mediated responses, including class I and class II responses. These methods include the use of adjuvants of various types, such as ISCOMS (Morein, *Immunology Letters* 25:281-284, 1990; Takahashi et al., *Nature* 344:873-875m, 1990), liposomes (Gergoriadis et al., *Vaccine* 5:145-151, 1987), lipid conjugation (Deres et al., *Nature* 342:561-564, 1989), coating of the peptide on autologous cells (Staerz et al., *Nature* 329:449-451, 1987), pinosomes (Moore et al., *Cell* 54:777-785, 1988), alum, complete or incomplete Freund's adjuvants (Hart et al., *Proc. Natl. Acad. Sci. USA* 88:9448-9452, 1991), or various other useful adjuvants (e.g., Allison and Byars, *Vaccines* 87:56-59, Cold Spring Harbor Laboratory, 1987) that allow effective parenteral administration (Litvin et al., *Advances in AIDS Vaccine Development*, Fifth Annual Meeting of the National Vaccine Development Groups for AIDS, August 30, 1992).

Alternatively, the proteins or peptides corresponding to the immunogenic portion(s) discussed above can be encapsulated for oral administration to elicit an immune response in enteric capsules (Chamock et al., *J. Amer. Med. Assoc.* 195:445-452, 1966) or other suitable carriers, such as poly (DL-lactide-co-glycolate) spheres (Eldridge et al. in *Proceedings of the International Conference on Advances in AIDS Vaccine Development*, DAIDS, NIAID, U.S. Dept of Health & Human Services, 1991) for gastrointestinal release.

As noted above, immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, *op. cit.*, Milich et al., *Proc. Natl. Acad. Sci. USA* 85:1610-1614, 1988; Willis, *Nature* 340:323-324, 1989; Griffiths et al., *J. Virol.* 65:450-456, 1991).

Within preferred embodiments of the present invention, pharmaceutical compositions are provided comprising one of the above described recombinant viruses, such as a recombinant retrovirus or recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles, corona and Sindbis virus in combination with a pharmaceutically acceptable carrier or diluent. The composition may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for either injection, oral, or rectal administration. Generally, the recombinant virus is utilized at a concentration ranging from 0.25% to 25%, and preferably about 5% to 20% before formulation. Subsequently, after preparation of the composition, the recombinant virus will constitute about 1 ug of material per dose, with about 10 times this amount material (10  $\mu$ g) as copurified contaminants. Preferably, the composition is prepared in 0.1-1.0 ml of aqueous solution formulated as described below.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a vector or recombinant virus in 10 mg/ml mannitol, 1 mg/ml HSA, 20mM Tris, pH 7.2 and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1  $\mu$ g of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months. The composition may be injected intravenously (*i.v.*) or subcutaneously (*s.c.*), although it is generally preferable to inject it intramuscularly (*i.m.*). The

individual doses normally used are  $10^7$  to  $10^9$  c.f.u. (colony forming units of neomycin resistance titrated on HT1080 cells). These are administered at one to four week intervals for three or four doses initially. Subsequent booster shots may be given as one or two doses after 6-12 months, and thereafter annually.

- 5 Oral formulations may also be employed with carriers or diluents such as cellulose, lactose, mannitol, poly (DL-lactide-co-glycolate) spheres, and/or carbohydrates such as starch. The composition may take the form of, for example, a tablet, gel capsule, pill, solution, or suspension, and additionally may be formulated for sustained release. For rectal administration, preparation of  
10 suppository may be accomplished with traditional carriers such as polyalkylene glucose, or a triglyceride.

- As noted above, the vector construct may direct expression of an immunomodulatory cofactor in addition to at least one immunogenic portion of a hepatitis antigen. If the vector construct, however, does not express an  
15 immunomodulatory cofactor which is a cytokine, this cytokine may be included in the above-described compositions, or may be administered separately (concurrently or subsequently) with the above-described compositions. Briefly, within such an embodiment, the immunomodulatory cofactor is preferably administered according to standard protocols and dosages as prescribed in *The*  
20 *Physician's Desk Reference*. For example, alpha interferon may be administered at a dosage of 1-5 million units/day for 2-4 months, and IL-2 at a dosage of 10,000-100,000 units/kg of body weight, 1-3 times/day, for 2-12 weeks. Gamma interferon may be administered at dosages of 150,000-1,500,000 U/m 2-3 times/week for 2-12 weeks.



The following examples are offered by way of illustration and not by way of limitation.

### EXAMPLES

5

#### EXAMPLE 1

##### ISOLATION OF HBV e/CORE SEQUENCE

- A 1.8 Kb BamH I fragment containing the entire precore/core coding region of hepatitis B is obtained from plasmid pAM6 (ATCC No 45020) and ligated into the BamH I site of KS II<sup>+</sup> (Stratagene, La Jolla, California). This plasmid is designated KS II<sup>+</sup> HBpc/c, Figure 1. Xho I linkers are added to the Stu I site of precore/core in KS II<sup>+</sup> HBpc/c and the resulting 877 base pair Xho I-Hinc II precore/core fragment is cloned into the Xho I/Hinc II site of SK II<sup>+</sup>. This plasmid is designated SK<sup>+</sup> HBe, Figure 1.

#### EXAMPLE 2

##### PREPARATION OF SEQUENCES UTILIZING PCR

##### 20 A. Site-Directed Mutagenesis of HBV e/core Sequences Utilizing PCR

- The precore/core gene in plasmid KS II<sup>+</sup> HB pc/c is sequenced to determine if the precore/core coding region is correct. This sequence was found to have a single base-pair deletion which causes a frame shift at codon 79 that results in two consecutive in-frame TAG stop codons at codons 84 and 85, Figure 2. This deletion is corrected by PCR overlap extension (Ho et al., *Gene* 77:51-59, 1989) of the precore/core coding region in plasmid SK<sup>+</sup> HBe. Four oligonucleotide primers are used for the 3 PCR reactions performed to correct the deletion.

- The first reaction utilizes two primers. The sense primer sequence corresponds to the nucleotide sequence 5 to 27 of the *adw* strain and contains two Xho I restriction sites at the 5' end. The nucleotide sequence numbering is obtained from Genbank (Intelligenics, Inc., Mountain View, California).

(SEQUENCE ID. NO. 1)

- 35 5'-3': CTC GAG CTC GAG GCA CCA GCA CCA TGC AAC TTT  
TT

The second primer sequence corresponds to the anti-sense nucleotide sequence 2158 to 2130 of the *adw* strain of hepatitis B virus and includes codons 79, 84 and 85.

5 (SEQUENCE ID. NO.2)

5'-3': CTA CTA GAT CCC TAG ATG CTG GAT CTT CC

The second reaction also utilizes two primers. The sense primer which corresponds to nucleotide sequence 2130 to 2158 of the *adw* strain and includes codons 79, 84 and 85.

10

(SEQUENCE ID. NO. 3)

5'-3': GGA AGA TCC AGC ATC TAG GGA TCT AGT AG

The second primer corresponds to the anti-sense nucleotide sequence from SK<sup>+</sup> plasmid polylinker downstream from codons 84 and 85.

15

(SEQUENCE ID. NO. 4)

5'-3': GGG CGA TAT CAA GCT TAT CGA TAC CG

The third reaction also utilizes two primers. The sense primer which corresponds to nucleotide sequence 5 to 27 of the *adw* strain and contains two Xho I restriction sites at the 5' end.

20

(SEQUENCE ID. NO. 1)

5'-3': CTC GAG CTC GAG GCA CCA GCA CCA TGC AAC TTT  
TT

25

The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK<sup>+</sup> plasmid polylinker downstream from codon 84 and 85.

(SEQUENCE ID. NO. 4)

30

5'-3': GGG CGA TAT CAA GCT TAT CGA TAC CG

The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81 (see Figure 2). Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second

35

PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence (Figure 3).

The PCR reactions are performed using the following cycling conditions: The sample is initially heated to 94°C for 2 minutes. This step, called the melting step, separates the double-stranded DNA into single strands for synthesis. The sample is then heated at 56°C for 30 seconds. This step, called the annealing step, permits the primers to anneal to the single stranded DNA produced in the first step. The sample is then heated at 72°C for 30 seconds. This step, called the extension step, synthesizes the complementary strand of the single stranded DNA produced in the first step. A second melting step is performed at 94°C for 30 seconds, followed by an annealing step at 56°C for 30 seconds which is followed by an extension step at 72°C for 30 seconds. This procedure is then repeated for 35 cycles resulting in the amplification of the desired DNA product.

The PCR reaction product is purified by gel electrophoresis and transferred onto NA 45 paper (Schleicher and Schuell, Keene, New Hampshire). The desired 787 bp DNA fragment is eluted from the NA 45 paper by incubating for 30 minutes at 65°C in 400 µl high salt buffer (1.5 M NaCl, 20mM Tris, pH 8.0, and 0.1mM EDTA). Following elution, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) is added to the solution. The mixture is vortexed and then centrifuged 14,000 rpm for 5 minutes. The aqueous phase, containing the desired DNA fragment, is transferred to a fresh 1.5 ml microfuge tube and 1.0 ml of 100% EtOH is added. This solution is incubated on dry ice for 5 minutes, and then centrifuged for 20 minutes at 10,000 rpm. The supernatant is decanted, and the pellet is rinsed with 500 µl of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum, and then resuspended in 10 µl deionized H<sub>2</sub>O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The 787 base pair Xho I-Cla I precore/core PCR amplified fragment is cloned into the Xho I-Cla I site of SK<sup>+</sup> plasmid. This plasmid is designated SK<sup>+</sup>HBc-c. *E. coli* (DH5 alpha, Bethesda Research Labs, Gaithersburg, Maryland) is transformed with the SK<sup>+</sup>HBc-c plasmid and propagated to generate plasmid DNA. The plasmid is then isolated and purified, essentially as described by Birnboim et al. (*Nuc. Acid Res.* 7:1513, 1979; see also *Molecular Cloning: A Laboratory Manual*, Sambrook et al. (eds.), Cold Spring Harbor Press, 1989). The SK<sup>+</sup>HB c-c plasmid is analyzed to confirm the sequence of the precore/core gene, Figure 4.

**B. Isolation of HBV core Sequence**

The single base pair deletion in plasmid SK<sup>+</sup> HBe is corrected by PCR overlap extension as described in Example 2A. Four oligonucleotide primers are used for the PCR reactions performed to correct the mutation.

- 5 The first reaction utilizes two primers. The sense primer corresponds to the nucleotide sequence for the T-7 promoter of SK<sup>+</sup>HBe plasmid.

(SEQUENCE ID. NO. 5)

- 10 5'-3': AAT ACG ACT CAC TAT AGG G

The second primer corresponds to the anti-sense sequence 2158 to 2130 of the *adw* strain, and includes codons 79, 84 and 85.

(SEQUENCE ID. NO. 2)

- 15 5'-3': CTA CTA GAT CCC TAG ATG CTG GAT CTT CC

The second reaction utilizes two primers. The anti-sense primer corresponds to the nucleotide sequence for the T-3 promoter present in SK<sup>+</sup>HBe plasmid.

- 20 (SEQUENCE ID. NO. 6)

5'-3': ATT AAC CCT CAC TAA AG

The second primer corresponds to the sense nucleotide sequence 2130 to 2158 of the *adw* strain, and includes codons 79, 84 and 85.

- 25 (SEQUENCE ID. NO. 3)

5'-3': GGA AGA TCC AGC ATC TAG GGA TCT AGT AG

The third reaction utilizes two primers. The anti-sense primer corresponds to the nucleotide sequence for the T-3 promoter present in SK<sup>+</sup>HBe plasmid.

30

(SEQUENCE ID. NO. 6)

5'-3': ATT AAC CCT CAC TAA AG

The second primer corresponds to the sense sequence of the T-7 promoter present in the SK<sup>+</sup>HBe plasmid.

35

(SEQUENCE ID. NO. 7)

5'-3': AAT ACG ACT CAC TAT AGG G

The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminates the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following primers:

The fourth reaction utilizes two primers. The sense primer corresponds to the nucleotide sequence 1885 to 1905 of the *adw* strain and contains two Xho I sites at the 5' end.

(SEQUENCE ID. NO. 8)

5'-3': CCT CGA GCT CGA GCT TGG GTG GCT TTG GGG  
15 CAT G

The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK<sup>+</sup> HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 3' end that was present in the multicloning site of SK<sup>+</sup> HBe plasmid.

(SEQUENCE ID. NO. 9)

5'-3': ATT ACC CCT CAC TAA AG  
25 Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3 M sodium acetate is added to this solution followed by 500  $\mu$ l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This  
30 solution is incubated at -20°C for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500  $\mu$ l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10  $\mu$ l deionized H<sub>2</sub>O. One microliter of the PCR product is analyzed by electrophoresis in a 1.5% agarose gel.  
35

### C. Isolation of HCV Core Sequences

A 200  $\mu$ L sample of serum is obtained from a patient with chronic non-A, non-B hepatitis and the viral RNA is prepared by the procedure of Christuano et al., *Hepatology* 14:51-55, 1991. The 200  $\mu$ L of serum is mixed with  
5 550  $\mu$ L of extraction buffer consisting of 4.2 M guanidinium isothiocyanate (Fluka Chemical Corp., St. Louis, Missouri), 0.5% sodium lauryl sarkosate and 25 mM Tris HCL, pH 8.0, and extracted once with phenol:chloroform (1:1), and once with chloroform. The aqueous phase is precipitated with an equal volume of isopropyl alcohol and centrifuged at 14,000 rpm for 5 minutes. The resulting pellet  
10 containing the viral RNA is washed with 70% ethanol and resuspended in 200  $\mu$ L of RNase-free deionized H<sub>2</sub>O. Four microliter of RNasin (40,000 U/ml) (Promega Corp., Madison, Wisconsin) is added to the mixture. This mixture contains the HCV RNA and is the template for the following reverse transcriptase reaction. Using the cDNA CYCLE kit (Invitrogen, San Diego, California) a full-  
15 length first strand cDNA is generated from the isolated viral mRNA. Seven microliters of the reverse transcription reaction above (100 ng of full-length first strand cDNA) is amplified by PCR in a total volume of 100  $\mu$ L of reaction mixture containing 10  $\mu$ L of 10 X PCR buffer (vial C16), 2  $\mu$ L of 25 mM dNTPs (vial C11), 5% DMSO, 4 U of Taq DNA polymerase (Cetus, Los Angeles, California) and 2  
20  $\mu$ M of each of the two primers.

The sense primer corresponds to the nucleotide sequence 316 to 335 and is the nucleotide sequence for the 5' region of the hepatitis C virus core open reading frame and includes the ATG start codon.

25 (SEQUENCE ID. NO. 10)

5'-3': GTA GAC CGT GCA TCA TGA GC

The second primer corresponds to the anti-sense nucleotide sequence 1172 to 1153 present in the hepatitis C virus envelope open reading frame.

30

(SEQUENCE ID. NO.12)

5'-3': ATA GCG GAA CAG AGA GCA GC

The reaction mixture is placed into a PCR Gene AMP System 9600 (Perkin-Elmer, Cetus, Los Angeles, California.). The PCR program regulates the  
35 temperature of the reaction vessel first at 95°C for 1 minute, then at 60°C for 2 minutes, and finally at 72°C for 2 minutes. This cycle is repeated 40 times.

Following the 40th cycle, the final cycle regulates the reaction vessel at 95°C for 1 minute, then at 67°C for 2 minutes, and finally at 72°C for 7 minutes.

In the first PCR reaction, the HCV core open reading frame from the 5' region upstream from the ATG start codon to the beginning of the HCV e open reading frame is amplified. The nucleotide numbering sequence is according to the HCV-J strain (Kato et al., *Proc. Natl. Acad. Sci. USA* 87:9524-9528, 1990).

The product from the first PCR reaction is amplified in a second PCR reaction. The second PCR amplification is performed with the sense primer that corresponds to the nucleotide sequence 329 to 367 (and is the nucleotide sequence for the 5' end of the hepatitis C virus core open reading frame). The 5' end of the sense primer contains two consecutive Xho I restriction sites. The primer also contains a number of nucleotide changes introduced in the area of the initiator ATG start codon to conform to appropriate rules for translation initiation (Kozak, *Mol. Biol.* 196:947-950, 1987).

15

(SEQUENCE ID. NO. 12)

5'-3': CTC GAG CTC GAG CCA CCA TGA GCA CAA ATC CTA  
AAC CTC AAA GAA AAA CCA AAC G

The anti-sense primer is designed to contain two consecutive stop codons in frame with HCV core gene. The 5' end of the primer contains two consecutive Hind III restriction sites. This primer corresponds to the nucleotide sequence 902 to 860, and is the junction between the hepatitis C virus core and envelope open reading frame.

25 (SEQUENCE ID. NO. 13)

5'-3': GC AAG CTT AAG CTT CTA TCA AGC GGA AGC TGG  
GAT GGT CAA ACA AGA CAG CAA AGC TAA GAG

The product from the first PCR reaction is also amplified in a third PCR reaction. The 5' end of the sense primer contains two consecutive Hind III restriction sites. This primer also contains nucleotide changes to conform to the Kozak rules for translation initiation and corresponds to the nucleotide sequence 329 to 367 of the HCV-J sequence (and is the nucleotide sequence for the 5' end of the hepatitis C virus core open reading frame).

35 (SEQUENCE ID. NO. 14)

5'-3': AAG CTT AAG CTT CCA CCA TGA GCA CAA ATC CTA  
AAC CTC AAA GAA AAA CCA AAC G

The anti-sense primer is designed to contain two stop codons in frame with the HCV core gene, and two consecutive Xho I restriction sites at the 5' end of the primer. This primer corresponds to the anti-sense nucleotide sequence 902 to 860, and is the junction between hepatitis C virus core and the envelope reading frame.

(SEQUENCE ID. NO. 15)

5'-3': GC CTC GAG CTC GAG CTA TCA AGA GGA AGC TGG  
GAT GGT CAA ACA AGA CAG CAA AGC TAA GAG

Using a TA Cloning Kit (Invitrogen, San Diego, California), the 570 bp PCR-amplified product from the second reaction is then ligated into the pCR II vector (Invitrogen, San Diego, California) and transformed into frozen competent *E. coli* cells. After verification by DNA sequencing this construct is designated pCR II Xh-H HCV core.

As described above, the 570 bp PCR amplified product from the third reaction is ligated into the pCR II vector. After verification by DNA sequencing this construct is designated pCR II H-Xh HCV core.

D. Isolation of HCV NS3/NS4 Sequence

The hepatitis C virus NS3/NS4 sequence is isolated from 200 µl of serum obtained from a patient with chronic non-A, non-B hepatitis as described in Example 1C. The viral RNA is reverse transcribed by the cDNA CYCLE Kit (Invitrogen, San Diego, California), and amplified by PCR. In the first PCR reaction, the HCV NS3/NS4 open reading frame is amplified.

The first PCR amplification is performed with two primers. The sense primer corresponds to the nucleotide sequence 3088 to 3106 of the hepatitis C virus NS2 open reading frame.

(SEQUENCE ID. NO. 16)

5'-3': GTG CAT GCA TGT TAG TGC G

The second primer corresponds to the anti-sense nucleotide sequence 6530 to 6511 of the hepatitis C virus NS5 open reading frame.

(SEQUENCE ID. NO. 17)

5'-3': CGT GGT GTA TGC GTT GAT GG

The product from the first PCR reaction is amplified in a second PCR reaction. The 5' end of the sense primer contains two consecutive Xho I



restriction sites. This primer also contains nucleotide changes to conform to the Kozak rules for translation initiation and corresponds to the nucleotide sequence 3348 to 3385 of the 5' region of the NS3 open reading frame of the HCV-J sequence.

5

(SEQUENCE ID. NO. 18)

5'-3': C CTC GAG CTC GAG CCA CCA TGG GGA AGG AGA  
TAC TTC TAG GAC CGG CCG ATA GTT TTG G

This primer corresponds to the nucleotide sequence 6368 to 6328 of  
10 HCV-J of the 3' region of the NS4 open reading frame of the HCV-J sequence.  
This primer contains two consecutive stop codons in frame with HCV core gene  
and two consecutive Hind III sites at its 5' end.

(SEQUENCE ID. NO. 19)

15

5'-3': GC AAG CTT AAG CTT CTA TCA GCG TTG GCA TGA  
CAG GAA AGG GAG TCC CGG TAA CCG CGG C

The 3020 bp PCR product from the second PCR reaction is ligated  
into the pCR II plasmid, verified by DNA sequencing and designated pCR II Xb-  
H HCV NS3/NS4.

20

#### E. Amplification of Immunomodulatory Cofactor IL-2

Jurkat cells are resuspended at  $1 \times 10^6$  cells/ml to a total volume of  
158 ml in T75 flasks. Phytohemagglutinin (PHA) is added to 1% of total volume  
(1.58 ml total), and incubated overnight at 37°C, 5% CO<sub>2</sub>. On the following day,  
25 cells are harvested in three 50 ml centrifuge tubes. The three pellets are  
combined in 50 ml PBS, centrifuged at 3,000 rpm for 5 minutes and supernatant  
decanted. This procedure is repeated. Poly A<sup>+</sup> mRNA is isolated using the  
Micro-Fast Track mRNA Isolation Kit, version 1.2 (Invitrogen, San Diego,  
California). The isolated intact mRNA is used as the template to generate full-  
30 length first strand cDNA by the cDNA CYCLE kit with the following primer.

This oligonucleotide corresponds to the anti-sense nucleotide  
sequence of the IL-2 mRNA, 25 base pairs downstream of the stop codon.

(SEQUENCE ID. NO. 20)

35

5'-3': ATA AAT AGA AGG CCT GAT ATG

The product from the reverse transcription reaction is amplified in  
two separate reactions. The first PCR amplification is performed with the sense

primer that corresponds to three bp upstream of the ATG start codon. This primer contains a Hind III site at its 5' end and contains the 5' region of the IL-2 open reading frame including the ATG start codon.

5 (SEQUENCE ID. NO. 21)

5'-3': GCA AGC TTA CAA TGT ACA GGA TGC AAC TCC  
TGT CT

The anti-sense primer is complementary to the 3' region of IL-2 open reading frame and starts three bp downstream of the TGA stop codon. This  
10 primer contains an Xho I site at the 5' end of the primer.

(SEQUENCE ID. NO. 22)

5'-3': GAC TCG AGT TAT CAA GTC AGT GTT GAG ATG  
ATG CT

15 The 467 bp PCR product from the first PCR reaction is ligated into the pCR II plasmid, verified by DNA sequencing and designated pCR II H-Xh IL-2

The product from the reverse transcription reaction is amplified in a second PCR reaction. The second PCR amplification is performed with the sense  
20 primer that corresponds to three bp upstream of the ATG start codon. This primer contains an Xho I site at its 5' end and contains the 5' region of the IL-2 open reading frame including the ATG start codon.

(SEQUENCE ID. NO. 23)

25 5'-3': GCC TCG AGA CAA TGT ACA GGA TGC AAC TCC  
TGT CT

The anti-sense primer is complementary to the 3' region of IL-2 open reading frame and starts three bp downstream of the TGA stop codon. This  
30 primer contains an Apa I site at the 5' end of the primer.

(SEQUENCE ID. NO. 24)

5'-3': GAG GGC CCT TAT CAA GTC AGT GTT GAG ATG  
ATG CT

35 The 467 bp PCR product from the second PCR reaction is ligated into the pCR II plasmid, verified by DNA sequencing and transformed into frozen competent *E. coli* cells. This vector construct is designated pCR II Xh-A IL-2.

F. Amplification of Immunomodulatory Cofactor B7/BB1 Utilizing PCR

Raji cells are suspended at  $1 \times 10^6$  cells/ml to a total volume of 158 ml in five T75 flasks and incubated overnight at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . On the following day, cells are harvested in three 50 ml centrifuge tubes. Cell pellets are combined in 50 ml PBS, centrifuged at 2,000 rpm for 10 minutes and supernatant decanted. This procedure is repeated. Poly A<sup>+</sup> mRNA is isolated as described in Example 2E. The isolated intact mRNA is used as the template to generate full-length first strand cDNA using the cDNA CYCLE kit, followed by two separate PCR amplification reactions essentially as described in Example 2E. The nucleotide numbering system is obtained from Freeman et al. (*J. Immunol.* 143:2714-2722, 1989).

The first PCR amplification is performed with two primers. The sense primer corresponds to the nucleotide sequence 315 to 353 of B7/BB1. This primer contains the 5' region of the B7/BB1 open reading frame including the ATG start codon and has two Hind III restriction sites at the 5' end.

(SEQUENCE ID. NO. 25)

5'-3': CG AAG CTT AAG CTT GCC ATG GGC CAC ACA CGG  
AGG CAG GGA ACA TCA CCA TCC

The second primer corresponds to the anti-sense nucleotide sequence 1187 to 1149 of B7/BB1. This primer is complementary to the 3' region of the B7/BB1 open reading frame ending at the TAA stop codon and contains two Xho I restriction sites at the 5' end.

(SEQUENCE ID. NO. 26)

5'-3': C CTC GAG CTC GAG CTG TTA TAC AGG GCG TAC  
ACT TTC CCT TCT CAA TCT CTC

The 868 bp PCR product from the first PCR reaction is ligated into the pCR II plasmid, verified by DNA sequencing and transformed into frozen competent *E. coli* cells. This vector construct is designated PCR II H-Xh-B7/BB1 and verified by DNA sequencing.

The second PCR amplification is performed with two primers. The sense primer corresponds to the nucleotide sequence 315 to 353 of B7/BB1. This primer contains the 5' region of the B7/BB1 open reading frame including the ATG start codon and has two Xho I sites at its 5' end.

(SEQUENCE ID. NO. 27)

5'-3': C CTC GAG CTC GAG GCC ATG GGC CAC ACA CGG  
AGG CAG GGA ACA TCA CCA TCC

5 The second primer corresponds to the anti-sense nucleotide sequence 1187 to 1149 of B7/BB1. This primer is complementary to the 3' region of the B7/BB1 open reading frame ending at the TAA stop codon and contains two Apa I restriction sites at the 5' end.

(SEQUENCE ID. NO. 28)

10 5'-3': C GGG CCC GGG CCC CTG TTA TAC AGG GCG TAC  
ACT TTC CCT TCT CAA TCT CTC

The 868 bp PCR product from the second PCR reaction is ligated into the pCR II plasmid, verified by DNA sequencing and transformed into frozen competent *E. coli* cells. This vector construct is designated pCR II Xh-A-B7/BB1  
15 and verified by DNA sequencing.

G. Synthesis of Immunomodulatory Cofactor GM-CSF Utilizing PCR

The synthesis of GM-CSF is performed following the protocol of Foguet and Lubbert (*Biotechniques* 13:674-675, 1992). Ten overlapping  
20 oligonucleotides, 53 to 106 nucleotides in length, are synthesized. The first oligonucleotide is the sense sequence of human GM-CSF from nucleotide sequence number 29 to 86 containing two Hind III cleavage sites at the 5' end.

(SEQUENCE ID. NO. 29)

25 5'-3': GCA AGC TTA AGC TTG AGG ATG TGG CTG CAG  
AGC CTG CTG CTC TTG GGC ACT GTG GCC TGC AGC ATC TCT GCA

The second oligonucleotide is the sense sequence of human GM-CSF from the nucleotide sequence numbers 29 to 86 containing two Xho I sites at the 5' end.

30

(SEQUENCE ID. NO. 47)

5'-3': GC CTC GAG CTC GAG GAG GAT GTG GCT GCA GAG  
CCT GCT GCT CTT GGG CAC TGT GGC CTG CAG CAT CTC TGC A

The third oligonucleotide is the anti-sense sequence of human GM-  
35 CSF from nucleotide sequence number 145 to 70.

(SEQUENCE ID. NO. 30)

5' -3': TCC TGG ATG GCA TTC ACA TGC TCC CAG GGC TGC  
GTG CTG GGG CTG GGC GAG CGG GCG GGT GCA GAG ATG CTG  
CAG

5 The fourth oligonucleotide is the sense sequence of human GM-CSF from nucleotide number 131 to 191.

(SEQUENCE ID. NO. 31)

5' -3': GAA TGC CAT CCA GGA GGC CCG GCG TCT CCT  
10 GAA CCT GAG TAG AGA CAC TGC TGC TGA GAT G

The fifth oligonucleotide is the anti-sense sequence of human GM-CSF from nucleotide number 282 to 176.

(SEQUENCE ID. NO. 32)

15 5' -3': CTT GTA CAG CTC CAG GCG GGT CTG TAG GCA  
GGT CGG CTC CTG GAG GTC AAA CAT TTC TGA GAT GAC TTC TAC  
TGT TTC ATT CAT CTC AGC AGC AGT

The sixth oligonucleotide is the sense sequence of human GM-CSF from nucleotide number 256 to 346.

20

(SEQUENCE ID. NO. 33)

5' -3': CCT GGA GCT GTA CAA GCA GGG CCT GCG GGG  
CAG CCT CAC CAA GCT CAA GGG CCC CTT GAC CAT GAT GGC CAG  
CCA CTA CAA GCA GCA CTG

25 The seventh oligonucleotide sequence is the anti-sense sequence of human GM-CSF from nucleotide number 331 to 389.

(SEQUENCE ID. NO. 34)

5' -3': GGT GAT AAT CTG GGT TGC ACA GGA AGT TTC  
30 CGG GGT TGG AGG GCA GTG CTG CTT GTA G

The eighth oligonucleotide is the sense sequence of human GM-CSF from nucleotide number 372 to 431.

(SEQUENCE ID. NO. 35)

35 5' -3': CAA CCC AGA TTA TCA CCT TTG AAA GTT TCA AAG  
AGA ACC TGA AGG ACT TTC TGC TTG TC

The ninth oligonucleotide sequence is the anti-sense sequence of human GM-CSF from nucleotide number 520 to 416 containing two Xho I restriction sites at the 5' end.

5 (SEQUENCE ID. NO. 36)

5'-3': GC CTC GAG CTC GAG GTC TCA CTC CTG GAC TGG  
CTC CCA GCA GTC AAA GGG GAT GAC AAG CAG AAA GTC C

The tenth oligonucleotide sequence is identical to oligonucleotide number nine except that it contains two Xba I restriction sites at the 5' terminus  
10 instead of Xho I restriction sites.

(SEQUENCE ID. NO. 37)

5'-3': GC TCT AGA TCT AGA GTC TCA CTC CTG GAC TGG  
CTC CCA GCA GTC AAA GGG GAT GAC AAG CAG AAA GTC C

15 All the oligonucleotides except for oligonucleotide Sequence ID Nos. 29, 36, 37 and 47 are phosphorylated. Ligation is performed by mixing 8 pmol of each oligonucleotide and 7.5  $\mu$ l 10X Sequenase Buffer (US Biochemical, Cleveland, Ohio) to a final volume of 75  $\mu$ l with sterile distilled deionized H<sub>2</sub>O. The reaction is heated for 5 minutes at 70°C, followed by 5 minutes at 48°C. Two  
20 microliters of dNTP mix (2.5mM each dNTP) and 10 U Sequenase are added and incubated for 30 minutes at 37°C. To inactivate the Sequenase, the ligation reaction is heated for 10 minutes at 70°C (*Current Protocols in Molecular Biology*, F.M. Asubel et al., 8.2.8-8.2.13, 1988).

25 One microliter of the ligation mixture is used in a PCR reaction with Vent polymerase (New England Biolabs, Beverly, Massachusetts) and the two oligonucleotides Sequence ID Nos. 29 and 36 as primers. The PCR product is ligated into the pCR II vector and transformed into frozen competent *E. coli* cells. This construct is designated pCR II H-Xh GM-CSF and verified by DNA sequencing.

30 One microliter of the ligation mixture was used in a second PCR reaction with Vent polymerase with the two oligonucleotides Sequence ID Nos. 47 and 37 as primers. The PCR product is ligated into the pCR II vector and transformed into frozen competent *E. coli* cells. This construct is designated pCR II Xh-Xb GM-CSF and verified by DNA sequencing.

35

#### H. Isolation of HBV Pre-S2 Open Reading Frame

The Pre-S2 open reading frame (including S) is PCR amplified with two primers and the pAM 6 plasmid (ATCC No. 45020). The sense primer corresponds to the nucleotides 3178 to 31 of the *adw* strain of hepatitis B virus.

- 5 The 5' end of the sense primer contains two consecutive Xho I restriction sites. The primer is the 5' region of the Pre-S2 open reading frame and includes the ATG start codon.

(SEQUENCE ID. NO. 49)

- 10 5'-3': GC CTC GAG CTC GAG GTC ATC CTC AGG CCA TGC  
AGT GGA ATT CCA CTG CCT TGC ACC AAG CTC TGC AGG

The second primer corresponds to the anti-sense nucleotide sequence 907 to 859 and contains two Cla I sites at the 5' end. This primer is complementary to the 3' region of the Pre-S2 open reading frame.

15

(SEQUENCE ID. NO. 49)

5'-3': GC ATC GAT ATC GAT GTT CCC CAA CTT CCA ATT  
ATG TAG CCC ATG AAG TTT AGG GAA TAA CCC C

- 20 The 957 bp PCR product is ligated into the pCR II plasmid, verified by DNA sequencing and designated pCR II HB-Pre-S2.

#### I. Isolation of HBV Polymerase Open Reading Frame

- The PCR amplification is performed with two primers and the pAM 6 plasmid (ATCC 40202). The sense primer corresponds to the nucleotides 2309 to 2370 of the *adw* strain of hepatitis B virus. The 5' end of the sense primer contains two consecutive Xho I restriction sites. This primer also contains nucleotide changes to conform to the Kozak rules for translation.

(SEQUENCE ID. NO. 50)

- 30 5'-3': GC CTC GAG CTC GAG ACC ATG CCC CTA TCT TAT  
CAA CAC TTC CGG AAA CTA CTG TTG TTA GAC GAC GGG ACC GAG  
GCA GG

- The second primer corresponds to the anti-sense nucleotide sequence 1645 to 1594 and contains two Cla I sites at the 5' end. This primer is complementary to the 3' region of the polymerase open reading frame and includes the TGA stop codon.
- 35

(SEQUENCE ID. NO. 51)

5'-3' GC ATC GAT ATC GAT GGG CAG GAT CTG ATG GGC  
GTT CAC GGT GGT CGC CAT GCA ACG TGC AGA GGT G

The 2564 bp PCR product is ligated into the pCR II plasmid,  
5 verified by DNA sequencing and designated pCR II HB-pol.

**J. Isolation of HBV ORF 5 Open Reading Frame**

The PCR amplification is performed with two primers and the pAM  
6 plasmid (ATCC 45020). The sense primer corresponds to the nucleotides 1432  
10 to 1482 of the *adw* strain of hepatitis B virus. The 5' end of the sense primer  
contains two consecutive Xho I restriction sites. The primer also contains  
nucleotide changes to conform to the Kozak rules for translation.

(SEQUENCE ID. NO. 52)

15 5'-3': GC CTC GAG CTC GAG ACC ATG TCC CGT CGG CGC  
TGA ATC CCG CGG ACG ACC CCT CTC GGG GCC GCT TGG GAC

The second primer corresponds to the anti-sense nucleotide  
sequence 1697 to 1648 and contains two Cla I sites at the 5' end. This primer is  
complementary to the 3' region of the ORF 5 open reading frame and includes the  
20 TAA stop codon.

(SEQUENCE ID. NO. 53)

5'-3': GC ATC GAT ATC GAT GGT CGG TCG TTG ACA TTG  
CTG GGA GTC CAA GAG TCC TCT TAT GTA AGA CC

25 The 293 bp PCR product is ligated into the pCR II plasmid, verified  
by DNA sequencing and designated pCR II HB-ORF 5.

**K. Isolation of HBV ORF 6 Open Reading Frame**

The PCR amplification is performed with two primers and the pAM  
30 6 plasmid (ATCC 45020). The sense primer corresponds to the nucleotides 1844  
to 1788 of the *adw* strain of hepatitis B virus. The 5' end of the sense primer  
contains two consecutive Xho I restriction sites. The primer also contains  
nucleotide changes to conform to the Kozak rules for translation.

35 (SEQUENCE ID. NO. 54)

5'-3': GC CTC GAG CTC GAG ACC ATG ATT AGG CAG AGG  
TGA AAA AGT TGC ATG GTG CTG GTG CGC AGA CCA ATT TAT GCC



The second primer corresponds to the anti-sense nucleotide sequence 1188 to 1240 and contains two Cla I sites at the 5' end. This primer is complementary to the 3' region of the ORF 6 open reading frame and includes the TAA

5

(SEQUENCE ID. NO. 55)

5'-3':GC ATC GAT ATC GAT GCT GAC GCA ACC CCC ACT  
GGC TGG GGC TTA GCC ATA GGC CAT CAG CGC ATG CG

The 687 bp PCR product is ligated into the pCR II plasmid, verified  
10 by DNA sequencing and designated pCR II HB-ORF 6.

### EXAMPLE 3

#### 15 A. Isolation of HBV X Antigen

A 612 bp Nco I-Sal I fragment containing the hepatitis B virus X open reading frame is obtained from the pAM6 plasmid (*adw*) (ATCC 45020), blunted by Klenow fragment, and ligated into the Hinc II site of SK<sup>+</sup> (Stratagene, La Jolla, California). This plasmid is designated SK-XAg.

20

*E. coli* (DH5 alpha, Bethesda Research Labs, Gaithersburg, Maryland) is transformed with the SK-XAg vector construct and propagated to generate plasmid DNA. The plasmid is then isolated and purified, essentially as described by Birnboim et al. (*Nuc. Acid Res.* 7:1513, 1979; *Molecular Cloning: A Laboratory Manual*, Sambrook et al. (eds.), Cold Spring Harbor Press, 1989).

25

#### B. Truncation of HBV X Antigen

In order to generate truncated XAg, the TAG stop codon is inserted by an in-frame deletion utilizing polymerase chain reaction (PCR) (Example 2C). The truncated X gene is then inserted into the Hinc II site of SK<sup>+</sup>. This plasmid  
30 is designated SK-DXAg. Mutants are confirmed using the assay described by Faktor et al. (*Oncogene* 5:867-872, 1990).

#### EXAMPLE 4

##### A. Preparation of Retroviral Backbone KT-3

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including gag sequences, from the N2 vector (Armentano et al., *J. Vir.* 61:1647-1650, 1987; Eglitis et al., *Science* 230:1395-1398, 1985) is ligated into the plasmid SK<sup>+</sup> (Stratagene, La Jolla, California). The resulting construct is designated N2R5. The N2R5 construct is mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT preventing gag expression. This mutagenized fragment is 200 base pairs (bp) in length and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the SK<sup>+</sup> plasmid and inserted into the Pst I site of N2 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is derived from pUC19 (Stratagene, La Jolla, California) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker. This construct is designated pUC31/N2R5gM.

A 1.0 Kilobase (Kb) MoMLV 3' LTR EcoR I-EcoR I fragment from N2 is cloned into plasmid SK<sup>+</sup> resulting in a construct designated N2R3<sup>+</sup>. A 1.0 Kb Cla I-Hind III fragment is purified from this construct. The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector (Kriegler et al., *Cell* 38:483, 1984; St. Louis et al., *PNAS* 85:3150-3154, 1988), comprising a SV40 early promoter driving expression of the neomycin phosphotransferase gene, is cloned into the SK<sup>+</sup> plasmid. A 1.3 Kb Cla I-BstB I gene fragment is purified from the SK<sup>+</sup> plasmid.

The expression vector is constructed by a three part ligation in which the Xho I-Cla I fragment containing the gene of interest and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5gM plasmid. The 1.3 Kb Cla I-BstB I neo gene fragment from the pAFVXM retroviral vector is then inserted into the Cla I site of this plasmid in the sense orientation.

##### B. Preparation of Retroviral Backbone KT-1

The KT-1 retroviral backbone vector is constructed essentially as described for KT-3 in Example 4A, with the exception that the dominant selectable marker gene, neo, is not inserted into the expression vector.

**EXAMPLE 5****CONSTRUCTION OF RETROVIRAL VECTORS**

5

**A. Construction of Hepatitis B Virus e-c Retroviral Vector**

The 787 bp Xho I-Cla I fragment from SK<sup>+</sup>HBc-c, Example 2A, is then ligated into the Xho I and Cla I sites of the KT-3 retroviral vector backbone. This construct is designated KT-HBc-e.

10

**B. Construction of Hepatitis B Virus core Retroviral Vector**

The PCR product from Example 2B, approximately 600 bp in length, is digested with Xho I and Cla I restriction endonucleases, electrophoresed through an 1.5% agarose gel and the DNA is purified from the gel slice by GeneClean II (Bio 101, Vista, California). This Xho I-Cla I HBV core PCR product is inserted into the Xho I and Cla I sites of the KT-3 retroviral vector backbone. The construct is designated KT-HBc.

15

The HBV core fragment (Xho I-Cla I) from KT-HBc is inserted into the respective sites of pBluescript KS<sup>+</sup> II (Stratagene, La Jolla, California). This construct is designated KS<sup>+</sup> II HBc, and is verified by DNA sequencing.

20

**C. Construction of Hepatitis C Virus core Retroviral Vector**

The Xho I-Hind III fragment from pCR II Xh-H HCV core is inserted into the respective sites of pSP72. This construct is designated pSP72 Xh-H HCc. The Xho I-Cla I fragment from pSP72 Xh-H HCc is then excised and inserted into the KT-3 backbone. This construct is designated KT-HCc.

25

**D. Construction of Hepatitis C Virus NS3/NS4 Retroviral Vector**

The Xho I-Hind III fragment from pCR II Xh-H HCV NS3/NS4 is inserted into the respective sites of pSP72. This construct is designated pSP72 Xh-H HCV NS3/NS4. The Xho I-Cla I fragment from pSP72 Xh-H HCV NS3/NS4 is then excised and inserted into the KT-3 backbone. This construct is designated KT-HCV NS3/NS4.

30

**E. Construction of Hepatitis B Virus x Retroviral Vector**

The SK-XAg open reading frame from Example 3A is checked for orientation by restriction enzyme analysis such that it is in the head to tail

35

orientation with respect to the Xho I and Cla I sites present in the SK<sup>+</sup> multicloning site. Then the X open reading frame from SK-XAg with the correct orientation is excised by Xho I and Cla I and inserted into the respective sites of the KT3 backbone. This construct is designated KT-HB-X.

5

**F. Construction of Hepatitis B Virus Pre-S2 Retroviral Vector**

The Xho I-Cla I fragment from PCR II Pre-S2 is excised and inserted into the respective sites of the KT3 backbone. This construct is designated KT-HB-Pre-S2.

10

**G. Construction of Hepatitis B Virus Polymerase Retroviral Vector**

The Xho I-Cla I fragment from pCR II HB-pol is excised and inserted into the respective sites of the KT3 backbone. This construct is designated KT-HB-pol.

15

**H. Construction of Hepatitis B Virus ORF 5 Retroviral Vector**

The Xho I-Cla I fragment from pCR II HB-ORF-5 is excised and inserted into the respective sites of the KT3 backbone. This construct is designated KT-HB-ORF 5.

20

**I. Construction of Hepatitis B Virus ORF 6 Retroviral Vector**

The Xho I-Cla I fragment from pCR II HB-ORF-6 is excised and inserted into the respective sites of the KT3 backbone. This construct is designated KT-HB-ORF 6.

25

**EXAMPLE 6**

**CONSTRUCTION OF MULTIVALENT RETROVIRAL VECTOR**

30

**A. Construction of Hepatitis B e/GM-CSF Retroviral Vector**

**i. Multivalent retroviral vector with IRBS**

pGEM 5Z<sup>+</sup>BIP 5' (Peter Sarnow, University of Colorado, Health Sciences Center, Denver, human immunoglobulin heavy chain binding protein) is digested with Sac I and Sph I. The 250 bp BIP fragment is isolate by 1.5% agarose gel electrophoresis and subcloned into the respective sites of pSP72. The vector construct is designated pSP72 BIP.

35

The Hind III-Xho I GM-CSF fragment is excised from pCR II H-Xh GM-CSF and subcloned into the Hind III-Xho I sites of pSP72 BIP. This construct is designated pSP72 BIP-GM-CSF.

The construct pSP72 BIP GM-CSF is cleaved at the Xho I site and  
5 blunted by Klenow fragment, followed by cleavage with Cla I. The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I restriction endonuclease. In a three-part ligation, the Xho I-Cla I fragment from SK<sup>+</sup> HBe-c, Example 2A, and the Cla I-blunted Xho I BIP-GM-CSF fragment is ligated into the Xho I-blunted Cla I sites of the KT-1  
10 retroviral backbone. This construct is designated KT-HBe-c/BIP-GM-CSF.

ii. *Multivalent retroviral vector with CMV promoter*

The 4.7 Kb CMV Env<sup>R</sup> Pst-RI fragment is isolated from pAF/CMV/Env<sup>R</sup> (U.S. Patent Application No. 07/395,932), and inserted into the  
15 Pst I and Eco RI sites of pUC 18. This construct is designated pUC 18 CMV Env<sup>R</sup>.

HIV-1 III<sub>B</sub> CAR is subcloned as a Sau 3A fragment from pAF/CMV/Env<sup>R</sup> into the BamH I site of pBluescript II KS<sup>+</sup> (Stratagene, La Jolla, California) to generate pBluescript II KS<sup>+</sup>/CAR. The CAR fragment  
20 is excised from pBluescript II KS<sup>+</sup>/CAR as a Xba I-Cla I fragment. The Xho I-Xba I HIV-1 III<sub>B</sub> gag/pol fragment is excised from SK<sup>+</sup> gag/pol SD delta (U.S. Patent Application No. 07/395,932). The plasmid backbone containing the CMV promoter is excised from pUC18 CMV/Env<sup>R</sup> with Xho I and Cla I. In a three part ligation, the Xho I-Xba I HIV III<sub>B</sub> gag-pol fragment and the Xba I-Cla I  
25 CAR fragment is inserted into the Xho I - Cla I sites of the pUC 18 CMV/Env<sup>R</sup> backbone to generate pUC 18 CMV gag/pol/CAR.

The Hind III-Xho I fragment containing the CMV IE promoter from pUC 18 CMV-gag/pol/CAR is subcloned into the respective sites of pCDNA II. This construct is designated pCDNA II CMV.

30 The Xho I-Xba I GM-CSF PCR product is subcloned from the pCR II Xh-Xb GM-CSF and inserted into the respective sites within pCDNA II-CMV. This construct is designated pCDNA II CMV-GM-CSF.

The pCDNA II CMV GM-CSF construct is cleaved at the Xba I site, blunted by Klenow fragment, followed by cleavage with Hind III. The KT-1  
35 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. In a three-part ligation, the Xho I-Hind III fragment from SK<sup>+</sup>HBe-c, Example 2A, and the Hind III-blunted Xba I CMV-GM-CSF

fragment is ligated into the Xho I-blunted Cla I sites of the KT-1 retroviral backbone. This vector construct is designated KT-HBe-c/CMV-GM-CSF.

**B. Construction of Hepatitis C core/IL-2 Retroviral Vector**

**5 i. Multivalent retroviral vector with IRBS**

The Hind III-Xho I IL-2 sequence is excised from pCR II H-Xh IL-2 and subcloned into the Hind III-Xho I sites of pSP72 BIP. This construct is designated pSP72 BIP IL-2. The Xho I-Hind III hepatitis C virus core sequence, Example 2C, is excised from pCRII Xh-H HCV C core and subcloned into the  
10 respective sites of pSP72. This construct is designated pSP72 Xh-H HCV core.

The construct pSP72 BIP-IL2 is cleaved at the Xho I site, blunted by Klenow fragment followed by cleavage with Eco RI. The Xho I-Eco RI HCV core fragment is isolated from pSP72 Xh-H HCV core. The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. In a  
15 three-part ligation, the Xho I-Eco RI HCV core fragment and the Eco RI-blunted Xho I BIP-IL2 fragment is ligated into the Xho I-blunted Cla I sites of the KT-1 retroviral backbone. This vector construct is designated KT-HCV core/BIP-IL2.

**ii. Multivalent retroviral vector with CMV promoter**

20 The Xho I-Apa I IL-2 fragment is excised from pCR II Xh-A IL-2 and subcloned into the respective sites of pCDNA II-CMV promoter. This construct is designated pCDNA II CMV-IL-2.

The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. The construct pCDNA II CMV-IL-2 is  
25 cleaved at the Apa I site, blunted by Klenow fragment and followed by cleavage with Hind III restriction endonuclease. In a three-part ligation, the Xho I-Hind III HCV core fragment from pCR II Xh-H HCV core and the Hind III-blunted Apa I CMV IL-2 fragment is ligated into the Xho I-blunted Cla I sites of the KT-1 retroviral backbone. This vector construct is designated KT-HCV core/CMV IL-

30 2.

**C. Construction of Hepatitis B core/B7/BB1 Retroviral Vector**

**i. Multivalent retroviral vector with IRBS**

The Hind III-Xho I B7/BB1 sequence is excised from pCR II H-Xh  
35 B7/BB1 and subcloned into the Hind III-Xho I sites of pSP72 BIP. This construct is designated pSP72 H-Xh BIP-B7/BB1.

The construct pSP72 H-Xh BIP-B7/BB1 is cleaved at the Xho I site, blunted by Klenow fragment followed by cleavage with Cla I. The Xho I-Cla I HBV core fragment is isolated from KS II<sup>+</sup> HBV core, Example 5B. The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. In a three-part ligation, the Xho I-Cla I HBV core fragment and the Cla I-blunted Xho I BIP-B7/BB1 fragment is ligated into the Xho I-blunted Cla I sites of the KT-1 retroviral backbone. This vector construct is designated KT-HBV core/BIP-B7/BB1, Example 8.

10        ii.    *Multivalent retroviral vector with CMV promoter*

The Xho I-Apa I B7/BB1 sequence is excised from pCR II Xh-A B7/BB1 and subcloned into the respective sites of pCDA II-CMV promoter. This construct is designated pCDNA II CMV-B7/BB1.

15        The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. The construct pCDNA II CMV-B7/BB1 is cleaved at the Apa I site blunted by Klenow fragment and followed by cleavage with HIND III restriction endonuclease. In a three-part ligation, the Xho I-Hind III HBV core fragment from KSII<sup>+</sup> HBV core and the Hind III-blunted Apa I CMV B7/BB1 fragment is ligated into the Xho I-blunted Cla I sites of the  
20        KT-1 retroviral backbone. This vector construct is designated KT-HBV core/CMV B7/BB1

D.    Construction of Hepatitis B e/Hepatitis C core Retroviral Vector

         i.    *Multivalent retroviral vector with IRBS*

25        The Hind III-Xho I HCV core PCR product is subcloned from the pCR II H-Xh HCV core, Example 2C, and inserted into the respective sites within pSP72-BIP. This construct is designated pSP72 BIP-HCV core.

30        The construct pSP72 BIP-HCV core is cleaved at the Xho I site, blunted by Klenow fragment, followed by cleavage with Cla I. The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. In a three part ligation, the Xho I-Cla I HBV e fragment from SK<sup>+</sup> HBe-c, Example 2A, and the Cla I-blunted Xho I BIP HCV core fragment is ligated into the Xho I-blunted Cla I sites of the KT-1 retroviral backbone. This vector construct is designated KT-HBV e/BIP HCV core.

35

ii. *Multivalent retroviral vector with CMV promoter*

The Xho I-Xba I HCV core fragment from pSP72 Xb-H HCV core (Example 6B i) is inserted into the respective sites of pCDNA II CMV plasmid. This construct is designated pCDNA II CMV HCV core.

- 5 The construct pCDNA II CMV HCV core is cleaved at the Xba I site, blunted by Klenow fragment, followed by cleavage with Hind III. The KT-1 backbone is cleaved by Cla I and blunted with Klenow fragment followed by cleavage with Xho I. In a three part ligation, the Xho I-Hind III HBV e sequence from SK<sup>+</sup>HBe-c, Example 2A, the Hind III-blunted Xba I CMV HCV core  
10 fragment is ligated into the Xho I-blunted Cla I sites of the KT-1 retroviral backbone. This vector construct is designated KT-HBV<sub>e</sub>/CMV HCV core.

EXAMPLE 7

15

TRANSIENT TRANSFECTION AND TRANSDUCTION OF  
PACKAGING CELL LINES HX AND DA

A. Plasmid DNA Transfection

- 20 DX cells (W092/05266) are seeded at  $5 \times 10^5$  cells on a 10 cm tissue culture dish on day 1 with Dulbecco's Modified Eagle Medium (DMEM) and 10% Fetal Bovine Serum (FBS). On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA co-precipitation is performed by mixing 40.0  $\mu$ l 2.5 M CaCl<sub>2</sub>, 10  $\mu$ g plasmid DNA and  
25 deionized H<sub>2</sub>O to a total volume of 400  $\mu$ l. Four hundred microliters of the DNA-CaCl<sub>2</sub> solution is added dropwise with constant agitation to 400  $\mu$ l precipitation buffer (50 mM HEPES-NaOH, pH 7.1; 0.25 M NaCl and 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of cells. The cells are  
30 incubated with the DNA precipitate overnight at 37°C. On day 3 the media is aspirated and fresh media is added. The supernatant containing virus is removed on day 4, passed through a 0.45  $\mu$  filter and used to infect the DA packaging cell line, murine fibroblasts or stored at -80°C.

35 B. Packaging Cell Line Transduction

DA (W092/05266) cells are seeded at  $5 \times 10^5$  cells/10 cm tissue culture dish in 10 ml DMEM and 10% FBS 4  $\mu$ g/ml polybrene (Sigma, St. Louis,



Missouri) on day 1. On day 2, 3.0 ml, 1.0 ml and 0.2 ml of the freshly collected virus containing DX media is added to the cells. The cells are incubated with the virus overnight at 37°C. On day 3 the media is removed and 1 ml DMEM, 10% FBS with 800 µg/ml G418 is added to the plate. Only cells that have been  
5 transfected with the vector and contain the neo selectable marker will survive. A G418 resistant pool is generated over a period of a week. The pool is tested for expression as described (Example 11). The pool of cells is dilution cloned by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/ml and adding 0.1 ml to each well (1cell/well) of  
10 a 96 well plate. Cells are incubated for 14 days at 37°C, 10% CO<sub>2</sub>. Twenty-four clones are selected and expanded up to 24 well plates, 6 well plates then 10 cm plates at which time the clones are assayed for expression and the supernatants are collected and assayed for viral titer.

The titer of the individual clones is determined by infection of  
15 HT1080 cells, human fibroblast cell line ATCC CCL 121. On day 1, 5x10<sup>5</sup> HT1080 cells are plated on each well of a 6 well microtiter plate in 3.0 ml DMEM, 10% FBS and 4 µg/ml polybrene. The supernatant from each clone is serially diluted 10 fold and used to infect the HT1080 cells in 1.0 ml aliquots. The cells are incubated with the vector overnight 37°C, 10% CO<sub>2</sub> and the media is replaced  
20 with fresh DMEM, 10% FBS media on day 2. On day 3, selection of transduced cells is performed by replacing the media with fresh DMEM, 10% FBS media containing 800 µg/ml G418. Cells are incubated at 37°C, 10% CO<sub>2</sub> for 14 days at which time G418 resistant colonies are scored at each dilution to determine the viral titer of each clone as colony forming units/ml (cfu/ml).

25 Using these procedures it can be shown that the titers of the HBVcore and HBVe producer cell lines are:

DAcore-1	8x10 <sup>5</sup> cfu/ml
DAcore-10	1x10 <sup>6</sup> cfu/ml
DAHBe 4-7	3x10 <sup>6</sup> cfu/ml

30 The packaging cell line HX, WO 92/05266, is transduced with vector generated from the DA vector producing cell line in the same manner as described for transduction of the DA cells from DX supernatant.

For transduction of the DA (WO 92/05266) cells with a multivalent vector, lacking a neo selectable marker, the infection procedure as noted above is  
35 used. However, instead of adding G418 to the cells on day 3, the cells are cloned by limiting dilution as explained above. Fifty clones are expanded for expression as explained above, and titer assayed as described in Example 9.

**EXAMPLE 8****DETECTION OF REPLICATION COMPETENT RETROVIRUSES**

5

The extended  $S^+L^-$  assay determines if replication competent, infectious virus is present in the supernatant of the cell line of interest. The assay is based on the empirical observation that infectious retroviruses generate foci on the indicator cell line  $MiCl_1$  (ATCC CCL 64.1). The  $MiCl_1$  cell line is derived from the Mv1Lu mink cell line (ATCC CCL 64) by transduction with Murine Sarcoma Virus (MSV). It is a non-producer, non-transformed, revertant clone containing a murine sarcoma provirus that forms sarcoma ( $S^+$ ) indicating the presence of the MSV genome but does not cause leukemia ( $L^-$ ) indicating the absence of replication competent virus. Infection of  $MiCl_1$  cells with replication competent retrovirus "activates" the MSV genome to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a  $0.45 \mu$  filter to remove any cells. On day 1 Mv1Lu cells are seeded at  $1 \times 10^5$  cells per well (one well per sample to be tested) of a 6 well plate in 2 ml DMEM, 10% FBS and 8  $\mu$ g/ml polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at  $37^\circ\text{C}$ , 10%  $\text{CO}_2$ . On day 2, 1.0 ml of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 ml of media. The positive control consists of three dilutions (200 focus forming units, (ffu), 20 ffu and 2 ffu each in 1.0 ml media) of MA virus (Miller et al., *Molec. and Cell. Biol.* 5:431-437, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3 the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13 the media on the Mv1Lu cells is aspirated and 2.0 ml DMEM and 10% FBS is added to the cells. In addition the  $MiCl_1$  cells are seeded at  $1 \times 10^5$  cells per well in 2.0 ml DMEM, 10% FBS and 8  $\mu$ g/ml polybrene. On day 14 the supernatant from the Mv1Lu cells is transferred to the corresponding well of the  $MiCl_1$  cells and incubated overnight at  $37^\circ\text{C}$ , 10%  $\text{CO}_2$ . On day 15, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. On day 21 the cells are examined under the microscope at 10X power for focus formation

(appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the  $MiCl_1$  cells.

5 Using these procedures, it can be shown that the HBV core producer cell lines DA core-1, DA core-10, and HBV<sub>e</sub> producer cell line DA HB<sub>e</sub> 4-7, are not contaminated with replication competent retroviruses.

#### EXAMPLE 9

10

##### TITERING OF MULTIVALENT VECTORS

Since the multivalent vectors do not contain a selectable marker, such as the neomycin gene, another way of titering the vector is described. More specifically, 1.0 ml of vector supernatant is diluted five fold to a final dilution of  $10^{-9}$  ml. One milliliter of each dilution is then used to transduce  $5 \times 10^5$  HT1080 cells (ATCC No. CCL 121) essentially as noted in Example 7B. However, instead of adding G418, DNA is extracted from each dish 7 days later as described by Willis (*J. Biol. Chem.* 259:7842-7849, 1984). The HBV e/core is amplified by PCR

15 20 using the following PCR primers obtained from Genset (Paris, France).

The PCR amplification for HBV e/core is performed with the sense primer that corresponds to the nucleotide sequence 1865 to 1889 of the *adw* clone.

(SEQUENCE ID. NO. 38)

25 5'-3': TTC AAG CCT CCA AGC TGT GCC TTG G

This primer corresponds to the anti-sense nucleotide sequence 2430 to 2409 of the *adw* clone.

(SEQUENCE ID. NO. 39)

30 5'-3': TCT GCG ACG CGG CGA TTG AGA

The probe sequence used to confirm the presence of the desired PCR product and corresponds to the nucleotide sequence 1926 to 1907 of the *adw* strain of hepatitis B virus.

35 (SEQUENCE ID. NO. 40)

5'-3': GGA AAG AAG TCA GAA GGC AA

The PCR amplification for hepatitis C core is performed with the sense primer that corresponds to the nucleotide sequence 328 to 342 of the HCV-J clone.

5 (SEQUENCE ID. NO. 41)

5'-3': CAT GAG CAC AAA TCC

This primer corresponds to the anti-sense nucleotide sequence 892 to 907 of the HCV-J clone.

10 (SEQUENCE ID. NO. 42)

5'-3': GGG ATG GTC AAA CAA G

The probe sequence used to confirm the presence of the desired 564 bp PCR product and corresponds to the nucleotide sequence 674 to 693 of the HCV-J clone.

15

(SEQUENCE ID. NO. 43)

5'-3': GTC GCG TAA TTT GGG TAA GG

The PCR amplification for hepatitis C NS3/NS4 is performed with the sense primer that corresponds to the nucleotide sequence 4876 to 4896 of the HCV-J clone.

20

(SEQUENCE ID. NO. 44)

5'-3': TCC TGT GTG AGT GCT ATG ACG

This primer corresponds to the anti-sense nucleotide sequence 6321 to 6302 of the HCV-J clone.

25

(SEQUENCE ID. NO. 45)

5'-3': GAA GTC ACT CAA CAC CGT GC

The probe sequence used to confirm the presence of the desired 1426 bp PCR product and corresponds to the nucleotide sequence 5618 to 5637 of the HCV-J clone.

30

(SEQUENCE ID. NO. 46)

5'-3': CAC ATG TGG AAC TTC ATC AG

The PCR products are analyzed by Southern blot analysis with the appropriate <sup>32</sup>P-labeled probes (Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY,

35

1989). Signal is expected in all of the lower dilutions and gradually decrease at higher dilutions. The last dilution where a signal is visible yields the infectious U/ml of the vector.

5

#### EXAMPLE 10

##### A. Transduction of Murine Cells with Vector Construct

The murine fibroblast cell lines BC10ME (ATCC No. TIB85) Bl6  
10 and L-M(TK<sup>-</sup>) (ATCC No. CCL 13) are grown in DMEM containing 4500 mg/L glucose, 584 mg/L L-glutamine (Irvine Scientific, Santa Ana, California) and 10% fetal bovine serum (FBS) (Gemini, Calabasas, California).

The BC10ME, Bl6, and L-M(TK<sup>-</sup>) fibroblast cell lines are plated at  $1 \times 10^5$  cells each in a 10 cm dish in DMEM, 10% FBS complete and 4  $\mu$ g/ml polybrene.  
15 Each is transduced with 1.0 ml of the retroviral vector having a vector titer of approximately  $10^5$  cfu/ml. Clones are selected in DMEM, 10% FBS and 800  $\mu$ g/ml G418 as described in Example 7B.

The EL4 (ATCC No. TIB 39) cells and EL4/A2/K<sup>b</sup> cells (Sherman, L. Scripps Institute, San Diego, California) are transduced by co-culture with the  
20 DA producer cells. Specifically,  $1.0 \times 10^6$  EL4 cells or  $1 \times 10^6$  EL4/A2/K<sup>b</sup> are added to  $1 \times 10^6$  irradiated (10,000 rads) DA (vector titer of approximately  $10^5$ - $10^6$ ) producer cells in RPMI 1640 (Irvine Scientific, Santa Ana, California), 10% FBS, and 4  $\mu$ g/ml polybrene (Sigma, St. Louis, Missouri) on day 1. On day 2,  $1.0 \times 10^6$  irradiated (10,000 rad) DA producer cells are added to the co-culture. On  
25 day 5 selection of the transduced EL4 or EL4/A2/K<sup>b</sup> cells is initiated with 800  $\mu$ g/ml G418. The pool is dilution cloned as described in Example 7B.

BC10ME, Bl6, L-M(TK<sup>-</sup>), EL-4 cells transduced by multivalent vectors are not selected in G418; they are cloned by limiting dilution as in Example 7B and assayed for expression as described in Example 11A.

30

##### B. Transduction of Human Cells with Vector Construct

Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed  
35 marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are transduced with retroviral vector expressing HBV core or e antigen. Transduction of LCL is accomplished by co-culturing  $1.0 \times 10^6$

LCL cells with  $1.0 \times 10^6$  irradiated (10,000 rads) HX producer cells in a 6 cm plate containing 4.0 ml of medium and 4.0 mg/ml polybrene. The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. After overnight co-culture at 37°C and 5% CO<sub>2</sub>, the LCL suspension cells are removed and  $1 \times 10^6$  cells are again co-cultured for another 6-18 hours in a fresh plate containing  $1.0 \times 10^6$  irradiated (10,000 rads) HX producer cells. Transduced LCL cells are selected by adding 800 mg/ml G418 and cloned to obtain high expression clones. The Jurkat A2/K<sup>b</sup> cells (L. Sherman, Scripps Institute, San Diego, California) are transduced essentially as described for the transduction of LCL cells. LCLs transduced by multivalent vectors are not selected in G418; they are cloned by limiting dilution as in Example 7B and assayed for expression as in Example 11A.

15.

#### EXAMPLE 11

##### EXPRESSION OF TRANSDUCE GENES

#### 20 A. ELISA

Cell lysates from cells transduced by KT-HBe or KT-HBc are made by washing  $1.0 \times 10^7$  cultured cells with PBS, resuspending the cells to a total volume of 600  $\mu$ l on PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350, (Fisher, Pittsburgh, Pennsylvania) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Core antigen and precore antigen in cell lysates and secreted e antigen in culture supernatant are assayed using the Abbott HBe, rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Illinois). Another sensitive EIA assay for precore antigen in cell lysates and secreted e antigen in culture supernatant is performed using the Incstar ETI-EB kit, (Incstar Corporation, Stillwater, MN). A standard curve is generated from dilutions of recombinant hepatitis B core and e antigen obtained from Biogen (Geneva, Switzerland).

Using these procedures approximately 10 ng/ml e antigen is expressed in transduced cell lines that have been prepared as described in paragraph 1 of this example (see Figure 5).

**B. Expression of Transduced Genes by Western Blot Analysis**

Proteins are separated according to their molecular weight (MW) by means of SDS polyacrylamide gel electrophoresis. Proteins are then transferred from the gel to a IPVH Immobilon-P membrane (Millipore Corp., Bedford, Massachusetts). The Hoefer HSI TTE transfer apparatus (Hoefer Scientific Instruments, California) is used to transfer proteins from the gel to the membrane. The membrane is then probed with polyclonal antibodies from patient serum that reacts specifically with the expressed protein. The bound antibody is detected using  $^{125}\text{I}$ -labeled protein A, which allows visualization of the transduced protein by autoradiography.

**C. Immunoprecipitation/Western Blot**

Characterization of the precore/core and e antigens expressed by transduced cells is performed by immunoprecipitation followed by Western blot analysis. Specifically, 0.5-1.0 ml of cell lysate in PBS or culture supernatant is mixed with polyclonal rabbit anti-hepatitis B core antigen (DAKO Corporation, Carpinteria, California) bound to G-Sepharose (Pharmacia LKB, Uppsala, Sweden) and incubated overnight at 4°C. Samples are washed twice in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA and boiled in sample loading buffer with 0.5% 2-beta mercaptoethanol. Proteins are transferred to Immobilon (Millipore Corp., Bedford, Maine) and probed with the DAKO polyclonal rabbit anti-hepatitis core antigen followed by  $^{125}\text{I}$ -protein A.

Using these procedures, it can be shown that the 17 Kd HB e protein is secreted by transduced mouse cells into the culture supernatant and the p22, p23 intermediate hepatitis B e products are present mainly the lysates of transduced mouse cells, Figure 6.

**EXAMPLE 12**

30

**A. Cytotoxicity Assays****i. Inbred Mice**

Six- to eight-week-old female Balb/c, C57Bl/6 and C3H mice (Harlan Sprague-Dawley, Indianapolis, Indiana) are injected twice intraperitoneally (i.p.) with  $1 \times 10^7$  irradiated (10,000 rads at room temperature) vector transduced BC10ME, Bl6 and L-M(TK<sup>-</sup>) cells respectively. Animals are sacrificed 7 days later and the splenocytes ( $3 \times 10^6/\text{ml}$ ) cultured *in vitro* with their

- respective irradiated transduced cells ( $6 \times 10^4$ /ml) in T-25 flasks (Corning, Corning, New York). Culture medium consists of RPMI 1640, 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50  $\mu$ g/ml gentamycin and  $10^{-5}$ M  $\beta$  2-mercaptoethanol (Sigma, St. Louis, Missouri). Effector cells are harvested 4-7 days later and tested using various effector:target cell ratios in 96 well microtiter plates (Corning, Corning, New York) in a standard chromium release assay. Targets are the transduced and non-transduced BC10ME, Bl6 and L-M(TK<sup>-</sup>) where the non-transduced cell lines are used as negative controls. Specifically,  $\text{Na}_2^{51}\text{CrO}_4$ -labeled (Amersham, Arlington Heights, Illinois)(100 uCi, 1 hr at 37°C) target cells ( $1 \times 10^4$  cells/well) are mixed with effector cells at various effector to target cell ratios in a final volume of 200  $\mu$ l. Following incubation, 100  $\mu$ l of culture medium is removed and analyzed in a Beckman gamma spectrometer (Beckman, Dallas, Texas). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as:  $[(\text{Effector cell} + \text{target CPM}) - (\text{SR})]/[(\text{MR}) - (\text{SR})] \times 100$ . Spontaneous release values of targets are typically 10%-20% of the MR.

ii. *HLA A2.1 Transgenic Mice*

- Six- to eight-week-old female HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Virginia) are injected twice intraperitoneally (i.p.) with  $1 \times 10^7$  irradiated (10,000 rads at room temperature) vector transduced EL4 A2/Kb cells. Animals are sacrificed 7 days later and the splenocytes ( $3 \times 10^6$ /ml) cultured *in vitro* with irradiated (10,000 rads) transduced Jurkat A2/K<sup>b</sup> cells ( $6 \times 10^4$ /ml) in flasks (T-25, Corning, Corning, New York). The remainder of the chromium release assay is performed as described in Example 12A, where the targets are transduced and non-transduced EL4 A2/K<sup>b</sup> and Jurkat A2/K<sup>b</sup> cells. Non-transduced cell lines are utilized as negative controls.

iii. *Human CTL assays*

- Human PBMC are separated by Ficoll (Sigma, St. Louis, Missouri) gradient centrifugation. Specifically, cells are centrifuged at 3,000 rpm at room temperature for 5 minutes. The PBMCs are restimulated *in vitro* with their autologous transduced LCL, Example 10B, at an effector:target ratio of 10:1 for 10 days. Culture medium consists of RPMI 1640 with prescreened lots of 5% heat-inactivated fetal bovine serum 1 mM sodium pyruvate and 50  $\mu$ m/ml gentamycin. The resulting stimulated CTL effectors are tested for CTL activity



using transduced autologous LCL or HLA matched cells as targets in the standard chromium release assay, Example 12A. Since most patients have immunity to EBV, the non-transduced EBV-transformed B-cells (LCL) used as negative controls, will also be recognized as targets by EBV-specific CTL along with the transduced LCL. In order to reduce high background cytotoxicity due to killing of labeled target cells by EBV-specific CTL, it is necessary to add unlabeled non-transduced LCL to labeled target cells at a ratio of 50:1.

#### B. Detection of Humoral Immune Response

- Humoral immune responses specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 µg/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with cells or direct vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain antibodies specific for HBV core or HBV e antigen.

Using these procedures, it can be shown that antibody to HBV core and e antigens can be induced, Figures 7A and 7B.

#### 25 C. T cell proliferation

- Antigen induced T-helper activity resulting from two or three injections of direct vector preparations expressing HBV core or e antigen, is measured *in vitro*. Specifically, splenocytes from immunized mice are restimulated *in vitro* of a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and  $10^{-5}$  2-beta mercaptoethanol at 37°C and 5% CO<sub>2</sub>, supernatant is tested for IL-2 activity, which is secreted specifically by T-helper cells stimulated by HBV core or e antigen. IL-2 activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214), which is dependent on IL-2 for growth. The CTLL-2 clone will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and are incubated at 37°C and 5%,

CO<sub>2</sub> for 3 days. Subsequently, 0.5  $\mu$ Ci <sup>3</sup>H-thymidine is added to the CTLL-2. <sup>3</sup>H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Massachusetts) and counted in a Beckman beta  
5 counter. The amount of IL-2 in a sample is determined from a standard curve generated from a standard recombinant IL-2 obtained from Boehringer Mannheim, Indianapolis, Indiana).

10

### EXAMPLE 13

#### IDENTIFICATION OF IMMUNOGENIC DOMAINS OF HBV PRECORE/CORE

T-cell epitopes may be predicted utilizing computer algorithms such  
15 as T-Sites (MedImmune, Maryland). From this analysis, peptides are synthesized and used to identify CTL epitopes. Effector cells from individuals with acute hepatitis B infection that have been stimulated *in vitro* with transduced autologous (Example 10B) LCL are tested on autologous LCLs coated with the peptide. The chromium release assay is performed as described in Example 12A iii, except that  
20 peptide is added to non-transduced Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>-labeled LCL along with effector cells to a final concentration of 1-100  $\mu$ g/ml. The reaction is incubated 4-6 hours and a standard chromium release assay performed as described in Example 12A i.

25

### EXAMPLE 14

#### TUMORIGENICITY AND TRANSFORMATION

##### A. Tumorigenicity Assay

30 Tumor formation in nude mice is a particularly important and sensitive method for determining tumorigenicity. Nude mice do not possess mature T-cells, and therefore lack a functional cellular immune system, providing a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if injected  
35 into nude mice. However, tumorigenic transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, the vector construct is administered by injection into nude mice. The mice are visually examined for a period of 4 to 16

weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present (Giovannella et al., *J. Natl. Cancer Inst.* 48:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of biological drugs," *Abnormal Cells*, New Products and Risk, Hopps and Petricciani (eds), Tissue Culture Association, 1985; Levenbook et al., *J. Biol. Std.* 13:135-141, 1985). This test is performed by Quality Biotech Inc., (Camden, New Jersey).

#### B. Transformation Assay

- 10 Tumorigenicity has shown to be closely correlated with the property of transformation. One assay which may be utilized to determine transformation in colony formation of cells plated in soft agar (MacPherson et al., *Vir.* 23:291-294, 1964). Briefly, one property of normal non-transformed cells is anchorage dependent growth. Normal non-transformed cells will stop proliferating when  
15 they are in semi-solid agar support medium, whereas transformed cells will continue to proliferate and form colonies in soft agar.

- HT1080 (ATCC CCL 121), a neoplastic cell line derived from human fibrosarcoma and known to cause tumors in 100% of nude mice, is used as the assay positive control. WI-38 (ATCC CCL 75), a diploid embryonic human  
20 lung cell line which is not tumorigenic in nude mice, is used as the assay negative control.

- WI-38 cell lines are transduced with the vector construct as described in Example 7B. Duplicate samples of each of the transduced cell lines, HT1080, and WI-38, are cultured in agar. Briefly, a lower layer of 5.0 ml 0.8%  
25 Bactoagar (Difco, Detroit, Michigan) in DMEM 17% FBS is set on 60 mm tissue culture plates. This is overlaid with 2.0 ml 0.3% Bactoagar in the same medium with the cells suspended at a concentration of  $5 \times 10^5$  cells/ml. To reduce background clumps, each cell line is strained through a 70  $\mu$ m nylon mesh before suspending in the agar solution. The plates are incubated at 37°C in a humidified  
30 atmosphere of 5% CO<sub>2</sub> for 14 days. Within 24 hours of plating, representative plates of each cell line are examined for cell clumps present at the time of plating. On day 13, the plates are stained with 1.0 ml INT viral stain (Sigma, St Louis, Missouri) and on day 14, they are scanned for colonies of 150  $\mu$ m in diameter using a 1 mm eyepiece reticle.

- 35 Only colonies spanning 150  $\mu$ m in any orientation are scored, because colonies of this size can be readily observed in all planes under the microscope and non-transformed cells rarely form colonies of this size. At the end

of the assay, the plating efficiencies for each cell line are calculated as  $b/a \times 100$ , where b equals the sum of colonies on all plates, and a equals the total number of cells plates. A non-transformed cell line is one which has a plating efficiency of lower than or equal to 0.001%. Therefore, a transformed cell line will have a  
5 plating efficiency of greater than 0.001% (Risser et al., *Virology* 59:477-489, 1974).

### EXAMPLE 15

10

#### ADMINISTRATION PROTOCOLS

##### A. Mice

The mouse system is used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of vector encoding  
15 HBV core or e antigen. Six- to eight-week-old female Balb/C, C57Bl6 or C3H mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) lyophilized HBV core or HBV e expressing retroviral vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. The chromium release CTL assays are  
20 preferred essentially as described in Example 12A i.

##### B. Chimpanzee Administration Protocol

The data generated in the mouse system from Example 15A is used to determine the protocol of administration of vector in chimpanzees chronically  
25 infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive three doses of vector encoding core or e antigen at 28 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of HBV-IT (V) formulation media. The dosage will be either  $10^6$  or  $10^7$  HBV-IT (V) cfu given in  
30 four 0.5 ml injections i.m. on each injection day. Blood samples will be drawn on days 4, 12, 24, 36, 52, 70 and 84 and months 6, 12, 18, 24, 30, and 36 in order to measure serum ALT levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen and to assess safety and tolerability of the treatment. The hepatitis B e antigen is detected by Abbott HB  
35 e rDNA EIA kit as described in Example 11A. Antibodies to HB e antigen can be detected by Abbott HB e rDNA EIA kit and efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 12A iii.

Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule will be determined for administration of the vector to subjects in human trials. There subjects are monitored for serum ALT levels, presence of hepatitis B e antigen and the presence of antibodies directed against the hepatitis B e antigen essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 12A.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Jolly, Douglas J.  
Chang, Stephen M.W.  
Lee, William T.L.  
Townsend, Kay  
O'Dea, Joann

(ii) TITLE OF INVENTION: HEPATITIS THERAPEUTICS

(iii) NUMBER OF SEQUENCES: 56

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Seed and Berry  
(B) STREET: 6300 Columbia Center, 701 Fifth Avenue  
(C) CITY: Seattle  
(D) STATE: Washington  
(E) COUNTRY: U.S.  
(F) ZIP: 98104

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: McMasters, David D.

- (B) REGISTRATION NUMBER: 33,963
- (C) REFERENCE/DOCKET NUMBER: 930049.407PC

(1x) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 206-622-4900
- (B) TELEFAX: 206-682-6031
- (C) TELEX: 3723836

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGAGCTCG AGGCACCAGC ACCATGCAAC TTTT

35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTACTAGATC CCTAGATGCT GGATCTTCC

29

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAAGATCCA GCATCTAGGG ATCTAGTAG

29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid



(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGCGATATC AAGCTTATCG ATACCG

26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AATACGACTC ACTATAGGG

19

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATTAACCCCTC ACTAAAG

17

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AATACGACTC ACTATAGGG

19

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTCGAGCTC GAGCTTGGGT GGCTTGGGG CATG

34

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTACCCCTC ACTAAAG

17

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTAGACCCTG CATCATGAGC

20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATAGCGGAAC AGAGAGCAGC

20

## (2) INFORMATION FOR SEQ ID NO:12:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCGAGCTCG AGCCACCATG AGCACAAATC CTAAACCTCA AAGAAAAACC AAACG

55

## (2) INFORMATION FOR SEQ ID NO:13:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCAAGCTTAA GCTTCTATCA AGCGGAAGCT GGGATGCTCA AACAAACAG CAAAGCTAAG 60

AG 62

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGCTTAAGC TTCCACCATG AGCACAAATC CTAACCTCA AAGAAAAACC AAACG 55

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 62 base pairs

(B) TYPE: nucleic acid

69

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCTCGAGCT CGAGCTATCA AGAGGAAGCT GGGATGGTCA AACAGACAG CAAAGCTAAG 60

AG 62

(2) INFORMATION FOR SEQ ID NO:16:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTGCATGCAT GTTAGTGCG

19

**(2) INFORMATION FOR SEQ ID NO:17:**

- (1) SEQUENCE CHARACTERISTICS:**
  - (A) LENGTH:** 20 base pairs
  - (B) TYPE:** nucleic acid
  - (C) STRANDEDNESS:** single
  - (D) TOPOLOGY:** linear

**(11) MOLECULE TYPE:** cDNA

**(111) HYPOTHETICAL:** NO

**(v) FRAGMENT TYPE:** N-terminal

**(x1) SEQUENCE DESCRIPTION: SEQ ID NO:17:**

CGTGGTGTAT GCGTTGATGG

20

**(2) INFORMATION FOR SEQ ID NO:18:**

- (1) SEQUENCE CHARACTERISTICS:**
  - (A) LENGTH:** 59 base pairs
  - (B) TYPE:** nucleic acid
  - (C) STRANDEDNESS:** single
  - (D) TOPOLOGY:** linear

**(11) MOLECULE TYPE:** cDNA

**(111) HYPOTHETICAL:** NO

**(v) FRAGMENT TYPE:** N-terminal

**(x1) SEQUENCE DESCRIPTION: SEQ ID NO:18:**



CCTCGAGCTC GAGCCACCAT GGGGAAGGAG ATACTTCTAG GACCGGCCGA TAGTTTTGG 59

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAAGCTTAA GCTTCTATCA GCGTTGGCAT GACAGGAAAG GGAGTCCCGG TAACCGCGGC 60

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATAAATAGAA GGCCTGATAT G

21

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCAAGCTTAC AATGTACAGG ATGCAACTCC TGTCT

35

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GACTCGAGTT ATCAAGTCAG TGTGAGATG ATGCT

35

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCCTCGAGAC AATGTACAAG ATGCAACTCC TGTCT

35

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAGGGCCCTT ATCAAGTCAG TGTGAGATG ATGCT

35

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGAAGCTTAA GCTTGCCATG GGCCACACAC GGAGGCAGGG AACATCACCA TCC

53

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCGAGCTC GAGCTGTTAT ACAGGGCGTA CACTTCCCT TCTCAATCTC TC

52

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 52 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCTCGAGCTC GAGGCCATGG GCCACACAG GAGGCAGGGA ACATCACCAT CC

52

(2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGGCCCGGG CCCCTGTTAT ACAGGGCGTA CACTTTCCT TCTCAATCTC TC

52

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 72 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

77

GCAAGCTTAA GCTTGAGGAT GTGGCTGCAG AGCCTGCTGC TCTGGGCAC TGTGGCCTGC 60  
AGCATCTCTG CA 72

## (2) INFORMATION FOR SEQ ID NO:30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TCCTGGATGG CATTACATG CTCCAGGGC TGGTGTGG GGCTGGGCGA GCGGGCGGGT 60  
GCAGAGATGC TGCAG 75

## (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAATGCCATC CAGGAGGCC GCGTCTCCT GAACCTGAGT AGAGACACTG CTGCTGAGAT 60  
G 61

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 96 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTTGACAGC TCCAGGCGGG TCTGTAGGCA GGTGGGCTCC TGGAGGTCAA ACATTCTGA 60  
GATGACTTCT ACTGTTTCAT TCATCTCAGC AGCAGT 96

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs



79

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTGGAGCTG TACAAGCAGG GCCTGCGGG CAGCCTCACC AAGCTCAAGG GCCCCTTGAC 60

CATGATGGCC AGCCACTACA AGCAGCACTG 90

(2) INFORMATION FOR SEQ ID NO:34:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGTGATAATC TGGGTTGCAC AGGAAGTTTC CGGGGTTGGA GGGCAGTGCT GCTTGTAG 58

## (2) INFORMATION FOR SEQ ID NO:35:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CAACCCAGAT TATCACCTTT GAAAGTTTCA AAGAGAACCT GAAGGACTTT CTGCTTGTC 59

## (2) INFORMATION FOR SEQ ID NO:36:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GCCTCGAGCT CGAGGTCTCA CTCCTGGACT GGCTCCCAGC AGTCAAAGGG GATGACAAGC 60  
AGAAAGTCC 69

## (2) INFORMATION FOR SEQ ID NO:37:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCTCTAGATC TAGAGTCTCA CTCCTGGACT GGCTCCCAGC AGTCAAAGGG GATGACAAGC 60  
AGAAAGTCC 69

## (2) INFORMATION FOR SEQ ID NO:38:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTCAAGCCTC CAAGCTGTGC CTTGG

25

(2) INFORMATION FOR SEQ ID NO:39:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TCTGCGACGC GGC&ATTGAG A

21

(2) INFORMATION FOR SEQ ID NO:40:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

83

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGAAAGAAGT CAGAAGGCAA

20

(2) INFORMATION FOR SEQ ID NO:41:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CATGAGCACA AATCC

15

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

84

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GGGATGGTCA AACAAAG

16

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTCGCGTAAT TTGGGTAAGG

20

## (2) INFORMATION FOR SEQ ID NO:44:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TCCTGTGTGA GTGCTATGAC G

21

## (2) INFORMATION FOR SEQ ID NO:45:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GAAGTCACTC AACACCGTGC

20

## (2) INFORMATION FOR SEQ ID NO:46:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CACATGTGGA ACTTCATCAG

20

## (2) INFORMATION FOR SEQ ID NO:47:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 72 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal



## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCCTCGAGCT CGAGGAGGAT GTGGCTGCAG AGCCTGCTGC TCTTGGGCAC TGTGGCCTGC 60  
AGCATCTCTG CA 72

## (2) INFORMATION FOR SEQ ID NO:48:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 68 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GCCTCGAGCT CGAGGTCATC CTCAGGCCAT GCAGTGAAT TCCACTGCCT TGCACCAAGC 60  
TCTGCAGG 68

## (2) INFORMATION FOR SEQ ID NO:49:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GCATCGATAT CGATGTTCCC CAACTTCAA TTATGTAGCC CATGAAGTTT AGGGAATAAC 60

CCC 63

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 79 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GCCTCGAGCT CGAGACCATG CCCCTATCTT ATCAACACTT CCGGAAACTA CTGTTGTTAG 60

ACGACGGGAC CGAGGCAGG 79

## (2) INFORMATION FOR SEQ ID NO:51:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 66 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GCATCGATAT CGATGGGCAG GATCTGATGG GCGTTCACGG TGATCGCCAT GCAACGTGCA 60  
GAGGTG 66

## (2) INFORMATION FOR SEQ ID NO:52:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GCCTCGAGCT CAGACCATG TCCGTCGGC GCTGAATCCC GCGGACGACC CCTCTCGGGG 60  
CCGCTTGGGA C 71

## (2) INFORMATION FOR SEQ ID NO:53:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GCATCGATAT CGATGGTCGG TCATTGACAT TGCTGGGAGT CCAAGAGTCC TCTTATGTAA 60  
GACC 64

## (2) INFORMATION FOR SEQ ID NO:54:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 74 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:54:

GCCTCGAGCT CGAGACCATG ATTAGGCAGA GGTGAAAAAG TTGCATGGTG CTGGTGCGCA 60  
GACCAATTTA TGCC .74

(2) INFORMATION FOR SEQ ID NO:55:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 67 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GCATCGATAT CGATGCTGAC GCAACCCCA CTGGCTGGGG CTTAGCCATA GGCCATCAGC 60  
GCATGCG 67

## (2) INFORMATION FOR SEQ ID NO:56:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 655 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CACCAGCAAC ATGCAACTTT TTCACCTCTG CCTAATCATC TCTTGTACAT GTCCCACTGT	60
TCAAGCCTCC AAGCTGTGCC TTGGGTGGCT TTGGGGCATG GACATTGACC CTTATAAAGA	120
ATTGGAGCT ACTGTGGAGT TACTCTGTT TTTGCCTTCT GACTTCCTTC CTCCGTCAG	180
AGATCTCCTA GACACCGCT CAGCTCTGTA TCGGGAAGCC TTAGAGTCTC CTGAGCATTG	240
CTCACCTCAC CACACCGCAC TCAGGCAAGC CATTCTCTGC TGGGGGAAT TGATGACTCT	300
AGCTACCTGG GTGGGTAATA ATTTGAAGA TCCAGCATCT AGGGATCTAG TAGTCAATTA	360
TGTTAATACT AACATGGGT TAAAAATTAG GCAACTATTG TGTTTCATA TATCTTGCCT	420
TACTTTTGA AGAGAGACTG TACTTGAATA TTGGTATCT TTCGAGTGT GGATTGCGAC	480
TCCTCCAGCC TATAGACCAC CAAATGCCCC TATCTTATCA ACACTCCGG AACTACTGT	540
TGTTAGACGA CGGGACCGAG GCAGTCCCC TAGAAGAAGA ACTCCCTCGC CTCGAGACG	600

WO 93/15207

PCT/US93/01009

93

CAGATCTCCA TCGCCGCGTC GCAGAAGATC TCAATCTCGG GAATCTCAAT GTTAG

655

Claims

1. A method of treating hepatitis B infections within warm-blooded animals comprising administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis B antigen, such that an immune response is generated.
2. A method of treating hepatitis in warm-blooded animals, comprising:
  - (a) administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis B antigen, such that an immune response is generated; and
  - (b) administering to the warm-blooded animal an immunomodulatory cofactor.
3. A method of treating hepatitis B infections in warm-blooded animals comprising administering to a warm-blooded animal a vector construct which co-expresses at least one immunogenic portion of a hepatitis B antigen and an immunomodulatory cofactor.
4. The method of claims 1 to 3 wherein said vector construct expresses HBcAg.
5. The method of claims 1 to 3 wherein said vector construct expresses HBsAg.
6. The method of claims 1 to 3 wherein said vector construct expresses an HBsAg.
7. The method of claim 6 wherein said HBsAg is selected from the group consisting of S, pre-S1 and pre-S2.
8. The method of claims 1 to 3 wherein said vector construct expresses the HBV pol antigen.



9. The method of claims 1 to 3 wherein said vector construct expresses ORF 5.

10. The method of claims 1 to 3 wherein said vector construct expresses ORF 6.

11. The method of claims 1 to 3 wherein said vector construct directs the expression of both HBsAg and HBcAg.

12. The method of claims 1 to 3 wherein said vector construct is carried by a recombinant retrovirus.

13. The method of claims 1 to 3 wherein said vector construct is carried by a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus, herpes virus, SV40, HIV, measles and Sindbis virus.

14. A vector construct which directs the co-expression of at least one immunogenic portion of a hepatitis B antigen and of an immunomodulatory cofactor.

15. A recombinant retrovirus carrying a vector construct according to claim 14.

16. A recombinant virus carrying a vector construct according to claim 14, said virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles and Sindbis virus.

17. A pharmaceutical composition comprising the recombinant retrovirus according to claim 15 in combination with a pharmaceutically acceptable carrier or diluent.

18. A pharmaceutical composition comprising the recombinant virus according to claim 16 in combination with a pharmaceutically acceptable carrier or diluent.

---

19. A method of destroying hepatitis B carcinoma cells comprising administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated.

20. A method of destroying hepatitis B carcinoma cells in a warm-blooded animal, comprising:

(a) administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated; and

(b) administering to the warm-blooded animal an immunomodulatory cofactor.

21. A method of destroying hepatitis B carcinoma cells comprising administering to a warm-blooded animal a vector construct which directs the co-expression of an immunogenic portion of antigen X and an immunomodulatory cofactor.

22. The method of claims 19 to 21 wherein said vector construct is carried by a recombinant retrovirus.

23. The method of claims 19 to 21 wherein said vector construct is carried by a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles and Sindbis virus.

24. A vector construct which directs the expression of an immunogenic portion of antigen X.

25. A vector construct which directs the expression of both an immunogenic portion of antigen X, and of an immunomodulatory cofactor.

26. A recombinant retrovirus carrying a vector construct according to claims 24 or 25.

27. A recombinant virus carrying a vector construct according to claims 24 or 25, said virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles and Sindbis virus.

28. A pharmaceutical composition comprising a recombinant retrovirus according to claim 26 in combination with a pharmaceutically acceptable carrier or diluent.

29. A pharmaceutical composition comprising a recombinant virus according to claim 27 in combination with a pharmaceutically acceptable carrier or diluent.

30. A method of treating hepatitis C infections in a warm-blooded animal comprising administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis C antigen, such that an immune response is generated.

31. A method of treating hepatitis C infections, comprising:

(a) administering to a warm-blooded animal a vector construct which directs the expression of at least one immunogenic portion of a hepatitis C antigen, such that an immune response is generated; and

(b) administering to the warm-blooded animal an immunomodulatory cofactor.

32. A method of treating hepatitis C infections in a warm-blooded animal comprising administering to a warm-blooded animal a vector construct which directs the co-expression of at least one immunogenic portion of a hepatitis C antigen and an immunomodulatory cofactor.

33. The method of claims 30 to 32 wherein said vector construct expresses the core antigen C.

34. The method of claims 30 to 33 wherein said vector construct expresses antigen E2/NS1.

35. The method of claims 30 to 33 wherein said vector construct expresses antigen NS2.

36. The method of claims 30 to 33 wherein said vector construct expresses antigen NS3.

37. The method of claims 30 to 33 wherein said vector construct expresses antigen NS4.

38. The method of claims 30 to 33 wherein said vector construct expresses antigen NS5.

39. The method of claims 30 to 33 wherein said vector construct is carried by a recombinant retrovirus.

40. The method of claims 30 to 33 wherein said vector construct is carried by a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles and Sindbis virus.

41. A vector construct which directs the expression of at least one immunogenic portion of a hepatitis C antigen.

42. A vector construct which directs the co-expression of at least one immunogenic portion of a hepatitis C antigen and of an immunomodulatory cofactor.

43. A vector construct which directs the co-expression of at least one immunogenic portion of a hepatitis B antigen and at least one immunogenic portion of a hepatitis C antigen.

44. A recombinant retrovirus carrying a vector construct according to claims 41, 42 or 43.

45. A recombinant virus carrying a vector construct according to claims 41, 42 or 43 said virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus,

parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles and Sindbis virus.

46. A pharmaceutical composition comprising a recombinant retrovirus according to claim 44 in combination with a pharmaceutically acceptable carrier or diluent.

47. A pharmaceutical composition comprising a recombinant virus according to claim 45 in combination with a pharmaceutically acceptable carrier or diluent.

48. A method of destroying hepatitis C carcinoma cells in warm-blooded animals comprising administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of the polyprotein antigen, such that an immune response is generated.

49. A method of destroying hepatitis C carcinoma cells in warm-blooded animals, comprising:

(a) administering to a warm-blooded animal a vector construct which directs the expression of an immunogenic portion of the polyprotein antigen, such that an immune response is generated; and

(b) administering to the warm-blooded animal an immunomodulatory cofactor.

50. A method of destroying hepatitis C carcinoma cells comprising administering a vector construct which co-expresses an immunogenic portion of the polyprotein antigen and an immunomodulatory cofactor.

51. The method of claims 48 to 50 wherein said vector construct is carried by a recombinant retrovirus.

52. The method of claims 48 to 50 wherein said vector construct is carried by a recombinant virus, said virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, adenovirus, parvovirus, herpes virus, and Sindbis virus.

53. A vector construct which directs the expression of an immunogenic portion of the polyprotein antigen.

54. A vector construct which directs the expression of both an immunogenic portion of the polyprotein antigen and of an immunomodulatory cofactor.

55. A recombinant retrovirus carrying a vector construct according to claims 52 or 53.

56. A recombinant virus carrying a vector construct according to claims 53 or 54, said virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus herpes virus, SV40, HIV, measles and Sindbis virus.

57. A pharmaceutical composition comprising a recombinant retrovirus according to claim 55 in combination with a pharmaceutically acceptable carrier or diluent.

58. A pharmaceutical composition comprising a recombinant virus according to claim 56 in combination with a pharmaceutically acceptable carrier or diluent.

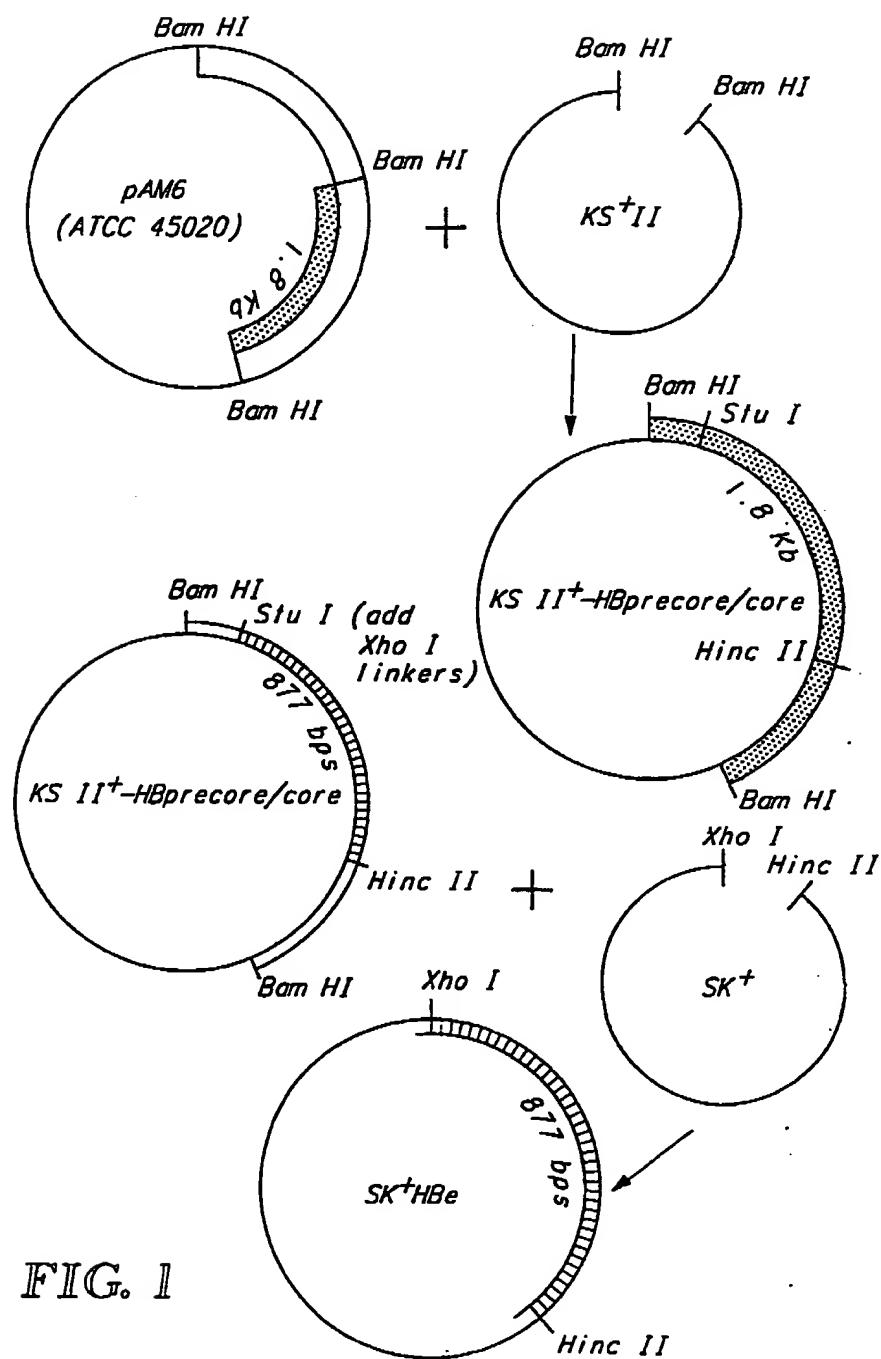


FIG. 1

SUBSTITUTE SHEET

C ACC AGC AAC

→ Precore

ATG CAA CTT TTT CAC CTC TGC CTA ATC ATC TCT TGT ACA TGT CCC

ACT GTT CAA GCC TCC AAG CTG TGC CTT GGG TGG CTT TGG GGC ATG → core

GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT ACT GTG GAG TTA CTC

TCG TTT TTG CCT TCT GAC TTC TTT CCT TCC GTC AGA GAT CTC CTA GAC

ACC GCC TCA GCT CTG TAT CGG GAA GCC TTA GAG TCT CCT GAG CAT

TGC TCA CCT CAC CAC ACC GCA CTC AGG CAA GCC ATT CTC TGC TGG GGG

GAA TTG ATG ACT CTA GCT ACC TGG GTG GGT AAT AAT TTG GAA GAT

79 81 34 85

CC G CAT CAA GGG ATC TAG TAG

CCA GCA TCT AGG GAT CTA GTA GTC AAT TAT GTT AAT ACT AAC ATG

GGT TTA AAA ATT AGG CAA CTA TTG TGG TTT CAT ATA TCT TGC CTT

ACT TTT GGA AGA GAG ACT GTA CTT GAA TAT TTG GTA TCT TTC GGA

GTG TGG ATT CGC ACT CCT CCA GCC TAT AGA CCA CCA AAT GCC CCT

ATC TTA TCA ACA CTT CCG GAA ACT ACT GTT GTT AGA CGA CGG GAC

CGA GGC AGG TCC CCT AGA AGA AGA ACT CCC TCG CCT CGC AGA CGC

AGA TCT CCA TCG CCG CGT CGC AGA AGA TCT CAA TCT CGG GAA TCT

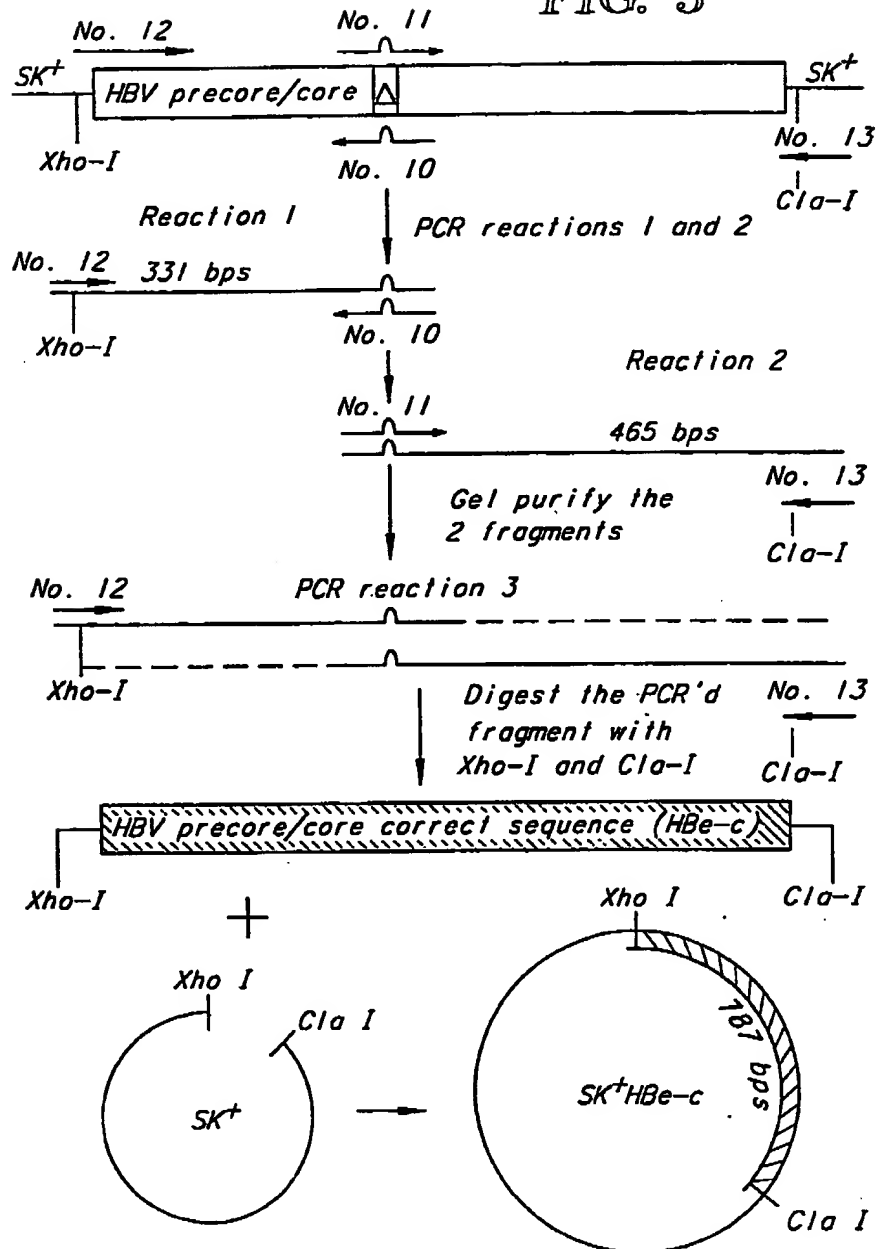
CAA TGT TAG

**Figure 2**

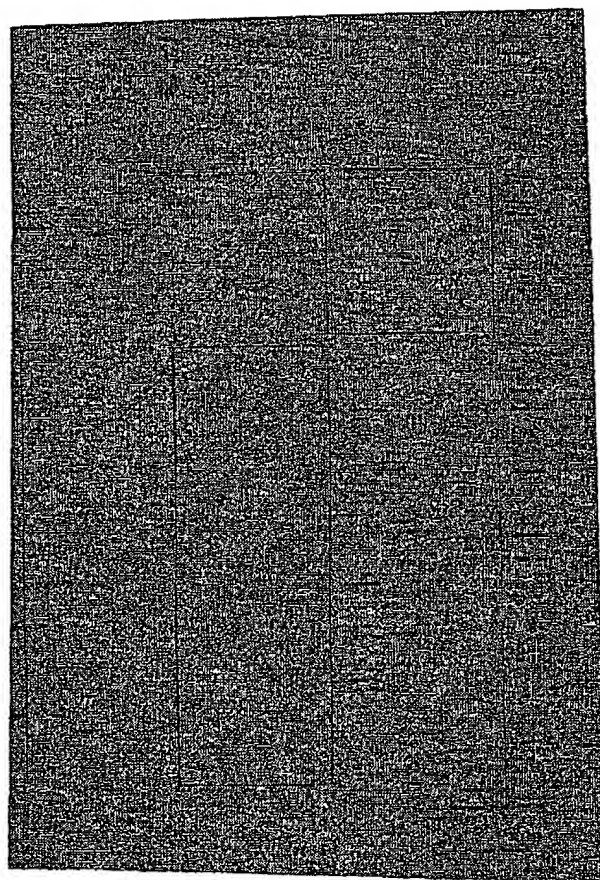
**SUBSTITUTE SHEET**



FIG. 3



SUBSTITUTE SHEET



**FIG. 4**

**SUBSTITUTE SHEET**

---

## FIG. 5

## ELISA for HBeAg

Standard: rHBeAg (Dave Milich/Biogen)

Sample-SN	<u>ng/ml</u>		Sample-Lysate	<u>ng/ml</u>
	Abbott	Incstar		Incstar
BC10ME	0.0	0.0		
BC/HBe 1-10	12.5	14.9		
BI/6	0.0	0.0		
BI/6/HBe 1-12	14.5	14.7		
LMTK	0.0	0.0	LMTK	0.0
LM/HBe 1-3	7.0	12.2	LM/HBe 1-3	6.0
LM/HBe 1-11	4.9	7.3	LM/HBe 1-11	4.5
LM/HBe 3-7	6.0	7.5	LM/HBe 3-7	9.8
LM/HBe 3-12	12.0	16.0	LM/HBe 3-12	16.0
JY-LCL	0.0	0.0		
JY/HBe 2-8	3.0	5.8		
EL4	0.0	0.0		
EL4/HBe 1-5	1.0	0.5		

SUBSTITUTE SHEET

FIG. 6

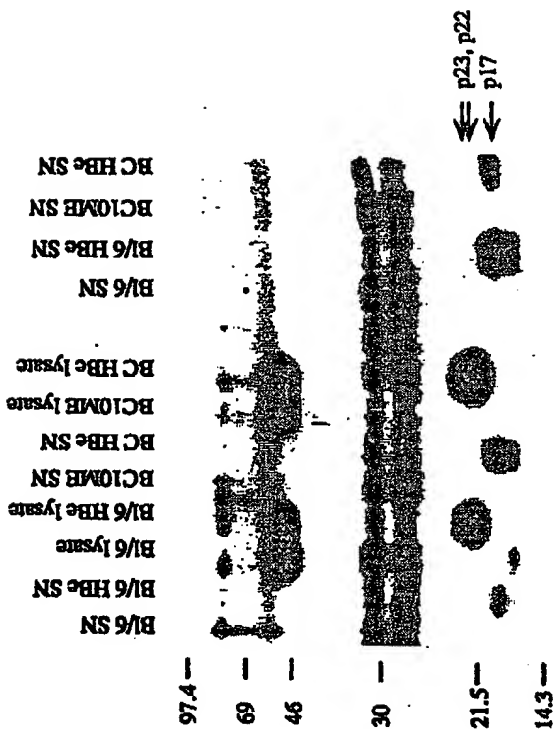
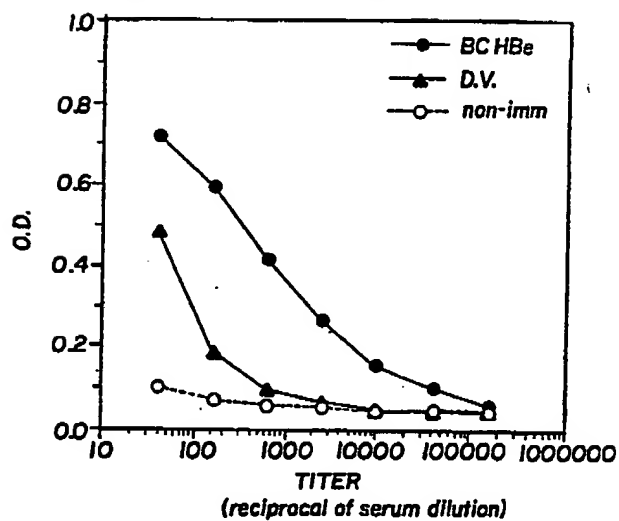
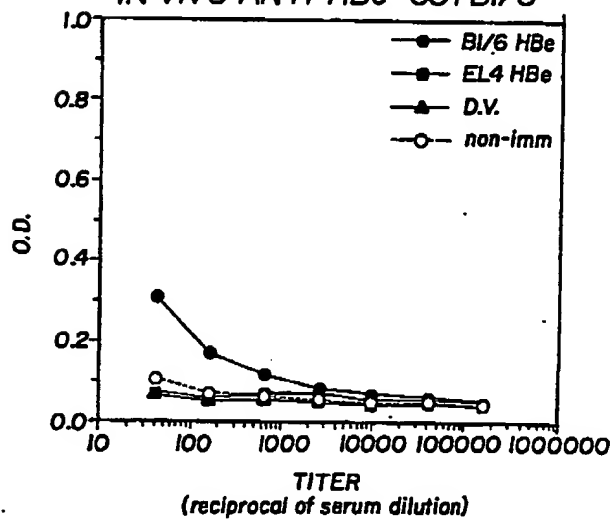


FIG. 7A

## IN VIVO ANTI-HBe BALB/c

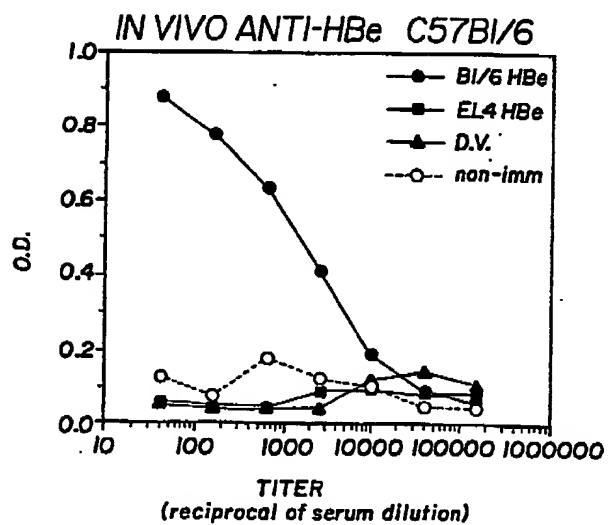
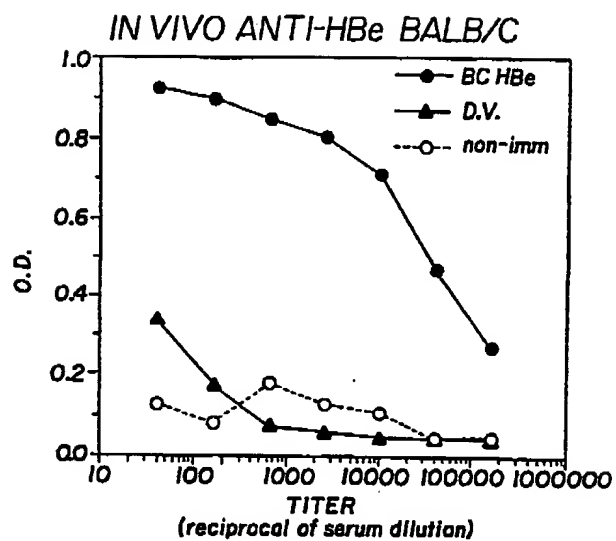


## IN VIVO ANTI-HBe C57BI/6



SUBSTITUTE SHEET

FIG. 7B



SUBSTITUTE SHEET

FIG. 8



SUBSTITUTE SHEET

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>8</sup> : <b>C12N 15/51, A61K 39/29</b> <b>C12N 15/86, 15/19</b>		A3	(11) International Publication Number: <b>WO 93/15207</b> (43) International Publication Date: <b>5 August 1993 (05.08.93)</b>
(21) International Application Number: <b>PCT/US93/01009</b> (22) International Filing Date: <b>4 February 1993 (04.02.93)</b> (30) Priority data: <b>07/830,417</b> <b>4 February 1992 (04.02.92)</b> <b>US</b> (71) Applicant: <b>VIAGENE, INC. [US/US]; 11075 Roselle Street, San Diego, CA 92121 (US).</b> (72) Inventors: <b>JOLLY, Douglas, J. ; 3050H Via Alicante Drive, La Jolla, CA 92037 (US). CHANG, Stephen, M., W. ; 9838 Via Caceras, San Diego, CA 92129 (US). LEE, William, Tsung-Liang ; 7961 Calle Posada, Carlsbad, CA 92009 (US). TOWNSEND, Kay ; 926 Birchview Drive, Encinitas, CA 92024 (US). O'DEA, Joann ; 8842 Cliff-ridge Avenue, LaJolla, CA 92037 (US).</b>		(74) Agents: <b>McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).</b> (81) Designated States: <b>AU, CA, CZ, JP, KP, KR, SK, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b> <i>Published</i> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (86) Date of publication of the international search report: <b>6 January 1994 (06.01.94)</b>	
(34) Title: <b>HEPATITIS THERAPEUTICS</b>			
(57) Abstract <p>The present invention provides a method of treating hepatitis B infections comprising the step of administering a vector construct which directs the expression of at least one immunogenic portion of a hepatitis B antigen, such that an immune response is generated. Also provided are methods for treating hepatitis C infections, as well as hepatocellular carcinomas.</p>			



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LJ	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/01009

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.C1.5	C 12 N 15/51	A 61 K 39/29 C 12 N 15/86
C 12 N 15/19		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.C1.5	C 12 N	A 61 K C 07 K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO, A, 8800971 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 11 February 1988	1-3, 13, 14, 16-18
Y	see page 4, line 23 - page 7, line 33	15
X	ARCH. VIROL. vol. 118, no. 1-2, 1991, pages 11 - 27 W.YE ET AL. 'Co-expression of hepatitis B virus antigens by a non-defective adenovirus vaccine vector' see the whole document	1, 4, 11
X	VIROLOGY vol. 168, no. 1, 1989, pages 31 - 39 A. RANEY ET AL. 'Retroviral mediated transfer and expression of hepatitis B e antigen' -/-	1, 4, 11, 12
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
11-08-1993	03. 12. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	Miss J. SKELLY	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	see the whole document	15
X	VIROLOGY vol. 170, no. 1, 1989, pages 99 - 106 O. JEAN-JEAN ET AL. 'Expression mechanism of hepatitis B virus C gene and biosynthesis of HBe antigen' see the whole document	1,4,11, 13
X	CHEMICAL ABSTRACTS, vol. 115, no. 23, 9 December 1991, Columbus, Ohio, US; abstract no. 249504, D. CHENG ET AL. 'Expression of hepatitis B virus C gene with different lenght of pre-core sequence by recombinant vaccinia viruses' page 225 ;column 2 ; see abstract & BINGDU XUEBAO vol. 7, no. 2, 1991, pages 170 - 175	1,4,11, 13

# INTERNATIONAL SEARCH REPORT

national application No.

PCT/US93/01009

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 1-4, 11-13 are directed to a method of treatment -  
of (diagnostic method practised on ) the human/ animal body the search has  
been carried out and based on the alleged effects of the compound/composi-  
tion.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such  
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see PCT/ISA/206 mailed on 10.09.93

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
(1-3) part. 4, 11, (12-18) part.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9301009  
SA 70119

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/11/93. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8800971	11-02-88	AU-B- 612983	25-07-91
		AU-A- 7789987	24-02-88
		EP-A- 0275300	27-07-88
		JP-T- 1500755	16-03-89
<hr/>			

EPV FORM 6000

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**THIS PAGE BLANK (USPTO)**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**